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(54) Title: INHIBITOR OF T CELL ACTIVATION

(57) Abstract: The present invention provides polynucleotides, polypeptides, pharmaceutical compositions, and methods for modulation of PKC θ activation, T-cell activation, NF- κ B activation, and supramolecular activation cluster assembly and localization.

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TITLE

Inhibitor of T-cell Activation

BACKGROUND OF THE INVENTION

5 A critical event in T-cell activation is the formation of a specialized junction between the T lymphocyte and the antigen-presenting cell. This interface has been termed the supra-molecular activation cluster, and consists of a central cluster of T-cell receptors surrounded by a ring of adhesion molecules. Three-dimensional analysis of this cluster has shown the presence of the θ isoform of protein kinase C, protein kinase C theta (PKC θ), in the central core region of the supra molecular activation cluster (SMAC), suggesting a key role for this kinase in T-cell activation (Monks et al., 1997, Nature 385:83-86; Monks et al., 1998, Nature 395:82-86). Subsequent in vitro and in vivo studies confirmed an essential role for PKC θ in transducing signals from the T-cell receptor leading ultimately to activation of transcription factors such as NF- κ B and AP-1 (Ghaffari-Tabrizi et al., 1999, Eur. J. Immunol. 29:132-42; Coudronniere et al., 2000, PNAS 97:3394-9; Khoshnan et al., 2000, J. Immunol. 165:6933-40; Werlen et al., 1998, EMBO J. 17:3101-11). Genetic inactivation of PKC θ demonstrated that T cells from PKC θ -deficient mice display a severe defect in CD3/CD28 induced T cell proliferation and more importantly, a complete absence of TCR, but not tumor necrosis factor alpha (TNF- α) or interleukin-1 (IL-1), initiated NF- κ B activation. Although these results indicate that PKC θ functions in a unique pathway that links the TCR signaling complex to the activation of NF- κ B in mature T cells, the molecular mechanism by which TCR activates PKC θ is still poorly understood.

25 Many protein kinases, including those of the protein kinase C family, require phosphorylation by upstream kinases in order to express full catalytic activity. Extensive biochemical, biophysical, and cell biological studies over many years have led to significant insight into how the activity of protein kinase C is regulated (Newtons, 1997, Curr. Opin. Cell Biol. 9:161-7; Newtons and Johnsons, 1998, Biochim. Biophys. Acta 1376:155-72). Protein kinases of this family contain a segment near the active site that must be phosphorylated in order to correctly align residues for catalysis and to stabilize the catalytically competent conformation of the

enzyme (Dutil and Newtons, 2000, JBC 275:10697-701). This segment, the activation loop, typically blocks the active site in an inactive conformation, and only moves out of the active site following phosphorylation. Phosphoinositide-dependent kinase PDK-1 has recently been shown to be the upstream kinase of a number of protein kinases including both atypical and conventional isoforms of PKC (Toker and Newtons, 2000, Cell 103:185-8). PDK-1 is regulated by its upstream kinase "phosphoinositide 3 kinase" (PI3 kinase) through the generation of lipid products such as phosphatidylinositol-3,4,5-triphosphate (Le Good et al., 1998, Science 281:2042-5). Prior studies have suggested the existence of a pathway where PI3-kinase-generated lipid products activate PDK-1, that in turn leads to the phosphorylation and activation of PKC family members. However, the effect of PDK-1 on PKC θ was not known, and the question remained whether the PI3 kinase/PDK-1 pathway could activate PKC θ in T-cells.

Presently, treatment of immunological diseases is not typically very specific, and often induces numerous side effects. For example, cancer drugs may be used to treat hyperproliferative immunological disorders, but many cancer drugs are not targeted to one type of cell. As a result, these drugs affect many different types of cells, both healthy and diseased. Cyclosporine is also used for immunosuppressive therapy, but the result of the use of this drug is a systemic immunosuppression, rendering the patient vulnerable to disease and illness. Accordingly, there is a long-felt need to identify and establish efficient, targeted methods of treating immunological diseases.

The protein kinase isoform PKC θ has been shown to play a critical role in T-cell activation and is essential for T-cell-receptor induced activation of the transcription factor NF- κ B. However, very little is known about the molecular events that lead to the activation of PKC θ in T-cells. Therefore, by establishing the sequence of events that lead to the activation of PKC θ upon T-cell receptor engagement, novel strategies can be identified for suppressing T-cell responses that could be used to develop directed therapies for immunological diseases.

BRIEF SUMMARY OF THE INVENTION

The present invention includes a polypeptide that interacts with phosphoinositide-dependent kinase-1 (PDK-1), methods of using the polypeptide, an isolated nucleic acid encoding the polypeptide, vectors containing the nucleic acid, and cells containing the vectors. The invention also includes a polypeptide that inhibits PKC θ activation, T-cell activation, NF- κ B activation, and prevents formation of the supramolecular activation cluster.

In one embodiment of the invention, an isolated nucleic acid encoding a peptide fragment of PKC θ , wherein the peptide fragment can selectively interact with PDK-1, is provided. Another embodiment of the invention provides the polypeptide encoded by the isolated nucleic acid, referred to herein as PIF θ .

An embodiment of the invention provides an isolated nucleic acid that is a homolog, variant, mutant or fragment of a nucleic acid encoding a peptide fragment of PKC θ , wherein the peptide fragment can selectively interact with PDK-1. Another embodiment of the invention provides the polypeptide encoded by the isolated nucleic acid that is a homolog, variant, mutant or fragment of a nucleic acid encoding a peptide fragment of PKC θ , wherein the peptide fragment can selectively interact with PDK-1.

One embodiment of the invention provides an isolated nucleic acid that is at least 90% identical to a nucleic acid encoding a peptide fragment of PKC θ , wherein the peptide fragment can selectively interact with PDK-1. Another embodiment of the invention provides the polypeptide encoded by the isolated nucleic acid that is at least 90% identical to a nucleic acid encoding a peptide fragment of PKC θ , wherein the peptide fragment can selectively interact with PDK-1.

In an aspect of the invention, the isolated nucleic acid encoding PIF θ comprises a fusion sequence encoding a membrane-permeabilizing sequence to facilitate transport of a peptide fragment/membrane-permeabilizing fusion protein into a cell. Another aspect of the invention provides the fusion protein encoded by the isolated nucleic acid encoding both PIF θ and a fusion sequence encoding a membrane-permeabilizing sequence to facilitate transport of a peptide fragment/membrane-permeabilizing fusion protein into a cell.

An embodiment of the invention provides a method that comprises modulating the activation of PKC θ in a cell by administering to a cell a fusion protein encoded by an isolated nucleic acid encoding a peptide fragment/membrane-permeabilizing sequence fusion protein.. Another embodiment of the invention provides a method wherein inhibition of the phosphorylation of PKC θ in the cell by administration of a peptide fragment/membrane-permeabilizing sequence fusion protein decreases the PKC θ activation. Yet another embodiment of the invention provides a method wherein activation of PKC θ is inhibited in the cell by a peptide fragment/membrane-permeabilizing sequence fusion protein.

In an aspect of the invention, a method is provided that comprises modulating the activation of NF- κ B in a cell by administering to a cell a fusion protein encoded by an isolated nucleic acid for a peptide fragment/membrane-permeabilizing sequence fusion protein. Another aspect of the invention provides a method wherein activation of a T-cell is modulated by administration of a peptide fragment/membrane-permeabilizing sequence fusion protein. In yet another aspect of the invention, a method is provided that comprises inhibiting T-cell activation by administering to a cell a fusion protein encoded by an isolated nucleic acid for a peptide fragment/membrane-permeabilizing sequence fusion protein. In another aspect of the invention, a method is provided that comprises inhibiting T-cell receptor-induced activation of a T-cell by administering to a mammal a fusion protein encoded by an isolated nucleic acid for a peptide fragment/membrane-permeabilizing sequence fusion protein.

In an embodiment of the invention, a method is provided for treating systemic lupus erythematosus, whereby the method comprises administering to a mammal a fusion protein encoded by an isolated nucleic acid for a peptide fragment/membrane-permeabilizing sequence fusion protein. In another embodiment of the invention, a method of suppressing the immune system of a organ-transplant patient comprises administering to the patient a fusion protein by an isolated nucleic acid for a peptide fragment/membrane-permeabilizing sequence fusion protein, such that the patient's immune system is suppressed.

An aspect of the invention provides a method for inhibiting PKC θ translocation to the supramolecular activation cluster in a T-cell by administering to a

mammal a fusion protein encoded by an isolated nucleic acid for a peptide fragment/membrane-permeabilizing sequence fusion protein. Another aspect of the invention provides a method of inhibiting formation of a supramolecular activation cluster in a T-cell by administering to a mammal a fusion protein encoded by an isolated nucleic acid for a peptide fragment/membrane-permeabilizing sequence fusion protein, whereby the components of the supramolecular activation cluster do not associate to form the supramolecular activation cluster.

In an embodiment of the invention, a composition is provided that includes a fusion protein encoded by an isolated nucleic acid for a peptide fragment/membrane-permeabilizing sequence fusion protein and a pharmaceutically-acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is an illustration of the PI3-kinase-PDK-1 pathway leading to the activation of several members of PKC family.

Figure 1B is a graph comparing Hela cells that were transiently transfected with pBIIx luciferase reporter gene along with PKC θ (0.4 pg) or p85 AN (0.4 μ g) either alone or in combination and then harvested after 24 hours and assayed for luciferase activity.

Figure 1C is a graph comparing purified T cells from KB-luciferase mice. Cells (1×10^6 cells/mL) were pre-treated with wortmannin (100 nM) or LY294002 (10 pM) for 30 min and then stimulated with plate-bound anti-CD3/CD28 antibodies (5 μ g/ml) for 18 hours.

Figure 1D is a graph comparing Hela cells that were transiently transfected with PKC θ wild type or mutants A148E (constitutively active; ca) or K409R (kinase dead; mut) (0.4 pg) in the presence or absence of PDK-1(0.4 μ g) and then harvested after 24 hours and assayed for luciferase activity.

Figure 1E is a graph comparing Hela cells that were transfected with PDK-1 wild type or mutants A277V (constitutively active; ca) or K111A/D223A (kinase dead; mut) (0.4pg) in the presence or absence of PKC θ (0.4 μ g) and then harvested after 24 hours and assayed for luciferase activity.

Figure 1F is a sequence alignment of both the activation loop and the

hydrophobic motif sequences for PKC isoforms.

Figure 1G is an illustration of the conserved activation loop Threonine (Thr538) and COOH-terminal Serine (Ser695) phosphorylation sites present in PKC θ .

5 Figure 1H is a series of immunoblots depicting purified T cells from B6 mice (2×10^6 cells/ml) that were stimulated with plate bound anti-CD3/CD28 antibodies (5 μ g/ml) for the indicated time. Cell lysate proteins were immunoblotted with the following antibodies: anti-phospho Thr538 of PKC θ , anti-pan phospho PKC θ (Cell signaling Technology, Beverly, MA) and anti-PKC θ (Transduction Lab, Lexington, KY).

10 Figure 1I is a graph comparing Hela cells that contained PKC θ wild type protein or mutants S695A or T538A (0.4 pg) in the presence or absence of PDK-1 (0.4 μ g) and then harvested after 24 hours and assayed for luciferase activity.

Figure 2A is an image of a series of immunoblots showing the levels of PKC θ and PDK-1 in HEK293 cell lysates after transfection with various PKC θ mutants.

15 Figure 2B is an illustration of PKC θ carboxyl terminal deletion mutants. Cells were transfected with PKC θ deletion mutants (1 μ g) and PDK-1 (1 pg) for 24 hours. After lysis, samples were immunoprecipitated by using anti-PDK-1 and immunoblotted with anti-PKC θ or anti-PDK-1. The expression level in lysates before immunoprecipitation was determined by immunoblotting with anti-PKC θ .

20 Figure 2C is a graph comparing Hela cells that were transfected for 24 hours with PKC θ deletion mutants alone (0.4 pg) or with PDK-1 (0.4 μ g) and then harvested after 24 hours and assayed for luciferase activity.

25 Figure 2D is a graph comparing Hela cells that were transfected with PKC θ , PIF θ , PDK-1 alone (0.4 pg) or with PKC θ and PDK-1 (0.4 pg) along with increasing amount (0.3-0.6-0.9 μ g) of PIF θ and then harvested after 24 hours and assayed for luciferase activity.

30 Figure 2E is an image of a series of immunoblots showing that PIF θ blocks the interaction of PKC θ with PDK-1 in 293 cells. Cells were transfected with PKC θ (1 μ g) and PDK-1 (1 μ g) along with increasing amount (0.5-1 pg) of GST-PIF θ for 24 hours. After lysis, samples were immunoprecipitated by using anti-PDK-1 and immunoblotted with anti-PDK-1 or anti-PKC θ . The expression level of GST-PIF θ in

lysates before immunoprecipitation was determined by immunoblotting with anti-GST.

Figure 2F is an image of a series of immunoblots showing that GST-PIF θ interacts with PDK-1 in 293 cells. Cells were transfected with GST-PIF θ and PDK-1 (1 μ g) for 24 hours. After lysis, samples were immunoprecipitated by using anti-GST and immunoblotted with anti-PDK-1 or anti-GST. The expression level PDK-1 in lysates before immunoprecipitation was determined by immunoblotting with anti-PDK-1.

Figure 2G is a series of images showing a schematic representation (left) and expression level of HA-tagged PDK-1 deletion mutants in 293 cells (right). Cells were transfected with GST-PIF θ (1 μ g) and HA-tagged PDK-1 deletion mutants (1 μ g) for 24 hours. After lysis, samples were immunoprecipitated by using anti-GST and immunoblotted with anti-HA. GST-PIF θ only interacted with the construct expressing the PDK-1 catalytic domain (PDK-1 84-332) (lower).

Figure 2H is a graph comparing Hela cells that were transfected with HA-tagged PDK-1 deletion mutants alone (0.4 μ g) or with PKC θ (0.4 μ g) and then harvested after 24 hours and assayed for luciferase activity.

Figure 3A is a diagram comparing sequences of wild-type and scrambled peptides indicating the Antennapodia homeodomain (lower case) and PKC θ segments.

Figure 3B is an image of a series of immunoblots demonstrating that PIF θ peptide inhibits PKC θ -PDK-1 interaction in primary T cells. Purified primary T cells from 136 mice were pre-treated with peptides for 3 hours and then stimulated with anti-CD3/CD28 plate bound antibodies for 1 hour. After lysis, samples were immunoprecipitated by using anti-PDK-1 and immunoblotted with anti-PKC θ or anti-PDK-1. The expression level in lysates before immunoprecipitation was determined by immunoblotting with anti-PKC θ .

Figure 3C is a series of immunoblots illustrating Jurkat cells that were incubated with 0.2, 2 or 20 μ M of each peptide followed by incubation with plate-bound anti-CD3/CD28 (5 μ g/ml) for 2 hours. EMSA was performed by using nuclear extracts and a specific KB-site probe as previously described.

Figure 3D is a graph illustrating luciferase reporter activity in cell

lysates for an assay in which purified T cells from KB-luciferase mice (1×10^6 cells/ml) were pre-treated with peptides for 3 hours and then stimulated with plate bound anti-CD3/CD28 antibodies ($5 \mu\text{g/ml}$) or TNF- α (100 ng/ml) for 18 hours.

5 Figure 3E is a graph illustrating purified T cells from B6 mice (5×10^5 cells/ml) that were pre-treated with peptides for 3 hours and then stimulated with plate bound anti-CD3/CD28 antibodies ($5 \mu\text{g/ml}$) with or without IL-2 (20 units/ml) for 48 hours. Thereafter the cells were incubated with MTT (5 mg/ml) for 4 hours and lysed to measure proliferation.

10 Figure 3F is a graph illustrating IL-2 production in supernatant of purified T cells from B6 mice (1×10^6 cells/ml) that were pre-treated with peptides for 3 hours and then stimulated with plate bound anti-CD3/CD28 antibodies ($5 \mu\text{g/ml}$) for 24 hours.

15 Figure 3G is a series of images illustrating surface expression of CD25 and CD69, assayed by flow cytometry 14 hours after stimulation with anti-CD3/CD25 antibodies ($5 \mu\text{g/ml}$). Thereafter the cells were collected and lysed to measure luciferase activity.

20 Figure 3H is a graph illustrating induction of CHS. The shaved mouse abdomen skin was painted with 20 ml of 0.5% DNFB (Sigma, St. Louis, MO) in acetone/olive oil (4:1). Five days later, mice were challenged, mice were challenged with 20 ml of 0.2% DNFB on each side of the right ear. As a control, the left ear was painted with an identical amount of vehicle. The ear thickness was measured at 24 hours after challenge. Mice treated with peptides received, 1 hour before the DNFB challenge, 1 mg of each peptide in 0.5 ml of PBS/0.005% DMSO.

25 Figure 4A is an image of an immunoblot illustrating that PI3-kinase inhibitors or PIF θ peptide inhibit the interaction of PKC θ with PI3-Kinase and LAT in Jurkat cells. Cells were pre-treated with wortmannin (100 nM) or LY294002 ($10 \mu\text{M}$) for 30 min and then stimulated with anti-CD3/CD28 plate bound anti-bodies for 1 hour. After lysis, samples were immunoprecipitated by using anti-PKC θ and immunoblotted with anti-PI3-kinase or anti-LAT.

30 Figure 4B is an image of an immunoblot illustrating that PI3-kinase inhibitors or PIF θ peptide inhibit the interaction of PKC θ with PI3-Kinase and LAT in Jurkat cells. Cells were pre-treated PIF peptides for 3 hours and then stimulated

with anti-CD3/CD28 plate bound anti-bodies for 1 hour. After lysis, samples were immunoprecipitated by using anti-PKC θ and immunoblotted with anti-PI3-kinase or anti-LAT.

Figure 4C is a series of images illustrating that PKC θ is localized to the cytoplasm in T-cells that were incubated with non-pulsed APCs and that PKC θ localized to the contact area between cells upon incubation with MCC-pulsed APCs. T cells were mixed with antigen-pulsed APC as previously described (Balamuth et al, 2001, Immunity 15:729-38). The expression level of PKC θ in lysates before immunoprecipitation was determined by immunoblotting with anti-PKC θ .

Figure 4D is a series of FITC-anti-Vb3-based immunodetection images illustrating that incubation of T-cells with MCC-pulsed APCs caused localization of the T-cell receptor and the receptor rafts to the supramolecular activation cluster, but did not cause localization with non-pulsed APCs. Pretreatment of T-cells with the wild type PIF θ peptide abolished supramolecular activation cluster formation, but scrambled PIF θ peptide had no effect.

Figure 4E is a series of FITC-conjugated cholera toxin-based immunodetection images illustrating that incubation of T-cells with MCC-pulsed APCs caused localization of the T-cell receptor and the receptor rafts to the supramolecular activation cluster, but did not cause localization with non-pulsed APCs. Pretreatment of T-cells with the wild type PIF θ peptide abolished supramolecular activation cluster formation, but scrambled PIF θ peptide had no effect.

DETAILED DESCRIPTION OF THE INVENTION

It has been discovered in the present invention that a fusion peptide consisting of PDK-1 Interacting Fragment of PKC θ (PIF θ) and a membrane-permeabilizing sequence can specifically block activation of PKC θ and NF- κ B activation in TCR-stimulated T-cells. Blocking the association of PKC θ with PDK-1 leads to the abolishment of synapse formation between T-cells and APC, suggesting a critical role for PKC θ in cellular events required for the formation of the supramolecular activation cluster (SMAC). More significantly, administration of the PIF θ peptide significantly attenuates immune responses in a mouse model of contact

hypersensitivity. The present invention establishes the sequence of events that leads to the activation of PKC θ upon TCR engagement and identifies a novel strategy for suppressing T-cell responses that is useful for development of future therapies for immunological diseases.

5 The present invention includes an isolated nucleic acid, isolated protein, and methods for the inhibition of full-length PKC θ activation, inhibition of T-cell activation, inhibition of supra molecular activation cluster (SMAC) formation and localization, and inhibition of NF- κ B activation. A key feature of the invention therefore is the design and expression of peptide fragments of PKC θ that inhibit such
10 formation and activation events. Further, isolated peptides expressed in this manner are a key feature of this invention.

 The present invention relates to peptide fragments of protein kinase C theta (PKC θ) and the use of these peptide fragments in the inhibition of full-length PKC θ activation, inhibition of T-cell activation, inhibition of supra molecular
15 activation cluster (SMAC) formation and localization, and inhibition of NF- κ B activation. The molecule responsible for activating PKC θ in T-cells is phosphoinositide-dependent kinase-1 (PDK-1). The specific region of interaction between PKC θ and PDK-1 has been mapped in the present invention and has been found to be localized to the C-terminus of PKC θ , now termed "PDK-1-interacting
20 fragment of PKC θ " (PIF θ).

 In one aspect of the invention, there is provided an isolated nucleic acid encoding a fragment of PKC θ . The invention should not be construed to be limited to the specific nucleic acid encoding a fragment of PKC θ disclosed herein, but rather, can include nucleic acids isolated from numerous sources, including
25 mammalian tissue and cDNA libraries. The isolated nucleic acid of the invention may be characterized using any technique well known in the art, such as nucleotide sequencing (Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York). Upon identification of the isolated nucleic acid as encoding a PKC θ polypeptide having the biological activity of interaction
30 with PDK-1, the isolated nucleic acid may be modified as described elsewhere herein.

 SEQ ID NO:1 is full-length human PKC θ cDNA (GenBank accession number NM-006257), and the corresponding protein is set forth in SEQ ID NO:2.

One aspect of the invention provides a truncated form of SEQ ID NO:2 (SEQ ID NO:3, which is encoded by the nucleic acid of SEQ ID NO:4) having only the carboxy-terminal portion of SEQ ID NO:2 to form a PKC θ protein fragment.

5 The invention should not be construed to be limited solely to a nucleic acid encoding a fragment of human PKC θ , but rather, should be construed to encompass any nucleic acid encoding a fragment of PKC θ , either known or unknown, which is capable of inhibiting T-cell activation, PKC θ translocation, and SMAC assembly when expressed. Modified nucleic acid sequences, i.e. nucleic acid sequences having sequences that differ from the nucleic acid sequences encoding the
10 naturally-occurring proteins, are also encompassed by the invention, so long as the modified nucleic acid still encodes a protein having the biological activity of modulating the growth of a neuron, for example. These modified nucleic acid sequences include modifications caused by point mutations, modifications due to the degeneracy of the genetic code or naturally occurring allelic variants, and further
15 modifications that have been introduced by genetic engineering, i.e., by the hand of man. Thus, the term nucleic acid also specifically includes nucleic acids composed of bases other than the five biologically occurring bases (adenine, guanine, thymine, cytosine and uracil).

Nucleic acids having at least 80 percent identity to SEQ ID NO:4 are
20 also encompassed by the present invention. More preferably, nucleic acids having at least 90 percent identity to SEQ ID NO:4 are also encompassed by the present invention. The determination of percent identity between two nucleotide or amino acid sequences can be accomplished using a mathematical algorithm. For example, a mathematical algorithm useful for comparing two sequences is the algorithm of
25 Karlin and Altschul (1990, Proc. Natl. Acad. Sci. USA 87:2264-2268), modified as in Karlin and Altschul (1993, Proc. Natl. Acad. Sci. USA 90:5873-5877). This algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, et al. (1990, J. Mol. Biol. 215:403-410), and can be accessed, for example at the National Center for Biotechnology Information (NCBI) world wide web site having the
30 universal resource locator <<<http://www.ncbi.nlm.nih.gov/BLAST/>>>. BLAST nucleotide searches can be performed with the NBLAST program (designated "blastn" at the NCBI web site), using the following parameters: gap penalty = 5; gap extension penalty = 2; mismatch penalty = 3; match reward = 1; expectation value

10.0; and word size = 11 to obtain nucleotide sequences homologous to a nucleic acid described herein. BLAST protein searches can be performed with the XBLAST program (designated "blastn" at the NCBI web site) or the NCBI "blastp" program, using the following parameters: expectation value 10.0, BLOSUM62 scoring matrix
5 to obtain amino acid sequences homologous to a protein molecule described herein. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al. (1997, Nucleic Acids Res. 25:3389-3402). Alternatively, PSI-Blast or PHI-Blast can be used to perform an iterated search which detects distant relationships between molecules (Id.) and relationships between
10 molecules which share a common pattern. When utilizing BLAST, Gapped BLAST, PSI-Blast, and PHI-Blast programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See <[<http://www.ncbi.nlm.nih.gov>>](http://www.ncbi.nlm.nih.gov).

In another aspect of the present invention, a nucleic acid encoding a fragment of PKC θ may have at least one nucleotide inserted into the naturally-
15 occurring nucleic acid sequence. Alternatively, a nucleic acid encoding a fragment of PKC θ may have at least one nucleotide deleted from the naturally-occurring nucleic acid sequence. Further, a nucleic acid encoding a fragment of PKC θ may have both a nucleotide insertion and a nucleotide deletion present in a single nucleic acid sequence encoding the protein.

20 Techniques for introducing changes in nucleotide sequences that are designed to alter the functional properties of the encoded proteins or polypeptides are well known in the art. Such modifications include the deletion, insertion, or substitution of bases, and thus, changes in the amino acid sequence. As is known to one of skill in the art, nucleic acid insertions and/or deletions may be designed into
25 the gene for numerous reasons, including, but not limited to modification of nucleic acid stability, modification of nucleic acid expression levels, modification of expressed polypeptide stability or half-life, modification of expressed polypeptide activity, modification of expressed polypeptide properties and characteristics, and changes in glycosylation pattern. All such modifications to the nucleotide sequences
30 encoding such proteins are encompassed by this invention.

It is not intended that the present invention be limited by the nature of the nucleic acid employed. The target nucleic acid may be native or synthesized nucleic acid. The nucleic acid may be DNA or RNA and may exist in a double-

stranded, single-stranded or partially double-stranded form. Furthermore, the nucleic acid may be found as part of a virus or other macromolecule. See, e.g., Fasbender et al., 1996, J. Biol. Chem. 272:6479-89.

5 The most preferred size of a PKC θ fragment of the invention is that of SEQ ID NO:4. However, an isolated nucleic acid of the present invention encoding a carboxy-terminal PKC θ fragment of more 14 amino acids (Met 686 to Pro 699) is also encompassed by the present invention.

10 In another aspect of the invention, a nucleic acid encoding a fragment of PKC θ is cloned into an expression vector downstream of the 3' end of a sequence encoding multiple functional tags. The amino-terminal fusion to PKC θ -encoding nucleic acid may comprise a multiple-histidine sequence to aid in purification of the expressed polypeptide, an epitope to aid in detection of the polypeptide, or a protease cleavage site for cleavage of the purification and detection sequences from the expressed polypeptide.

15 In another aspect of the invention, PKC θ may be cloned from a Jurkat T-cell.

20 An isolated nucleic acid of the present invention may be cloned into a DNA vector. A vector is a replicable DNA construct, used either to amplify DNA encoding the polynucleotide of the invention and/or to express a polypeptide encoded by the polynucleotide of the invention. An expression vector is a replicable DNA construct in which the polynucleotide of the invention is operably linked to suitable control sequences capable of effecting the expression of polypeptide in a suitable host. The need for such control sequences will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include a
25 transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation. Amplification vectors do not require expression control domains. All that is needed is the ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate
30 recognition of transformants.

In yet another aspect of the present invention, a nucleic acid encoding a fragment of PKC θ may be expressed in mammalian cells, using an appropriate

expression vector and mammalian cell. However, as evidenced by the literature relevant to the art, one skilled in the art will appreciate that a PKC θ -encoding nucleic acid can also be expressed in other eukaryotic cells, including yeast, or in prokaryotic cells, including bacteria. A PKC θ protein fragment of the present invention may be
5 expressed using any technique well-known in the art, such as simple expression, high level expression, or overexpression (Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York).

In another aspect of the present invention, PKC θ may be cloned from a Jurkat T-cell line into a pcDNA3 FLAG vector. In still a further aspect of the present
10 invention, PKC θ may be cloned from a Jurkat T-cell line into a pCMV FLAG vector.

The nucleic acids of the invention may be purified by any suitable means, as are well known in the art. For example, the nucleic acids can be purified by reverse phase or ion exchange HPLC, size exclusion chromatography or gel
15 electrophoresis. Of course, the skilled artisan will recognize that the method of purification will depend in part on the size of the DNA to be purified.

The present invention relates to methods of using polypeptides encoded by the isolated nucleic acids of the invention. Such methods may be used to modulate the activity or level of PKC θ in a cell. The present invention also relates to
20 methods of using polypeptides encoded by the nucleic acids of the invention to modulate the activation of NF- κ B and T-cells, and to inhibit the formation of the supramolecular activation cluster. The present invention further relates to methods of using polypeptides encoded by nucleic acids of the invention to treat a patient with systemic lupus erythematosus (SLE), and to suppress the immune system of a patient. It will be understood that these methods may use full length nucleic acids encoding
25 polypeptides of the invention, or homologs, variants, mutants or fragments thereof.

SEQ ID NO:2 illustrates the full-length human PKC θ polypeptide. SEQ ID NO:3, MDQNMFRNFSFMNP, illustrates a truncated form of PKC θ of the present invention. The truncated form of PKC θ has the amino acid sequence from
30 Met 1 to Ser 685 deleted from the amino-terminus of the polypeptide and Gly 700 to Ser 706 deleted from the carboxy-terminus of the polypeptide.

The present invention also provides for analogs of proteins or peptides encoded by PKC θ nucleic acids. Analogs can differ from naturally occurring proteins

or peptides by conservative amino acid sequence differences or by modifications which do not affect sequence, or by both. One embodiment of the present invention provides a peptide with at least 80 percent, and more preferably, at least 90 percent identity to SEQ ID NO:3.

5 For example, conservative amino acid changes may be made, which although they alter the primary sequence of the protein or peptide, do not normally alter its function. Conservative amino acid substitutions typically include substitutions within the following groups:

 glycine, alanine;
10 valine, isoleucine, leucine;
 aspartic acid, glutamic acid;
 asparagine, glutamine;
 serine, threonine;
 lysine, arginine;
15 phenylalanine, tyrosine.

 Modifications (which do not normally alter primary sequence) include in vivo, or in vitro chemical derivatization of polypeptides, e.g., acetylation, or carboxylation. Also included are modifications of glycosylation, e.g., those made by modifying the glycosylation patterns of a polypeptide during its synthesis and
20 processing or in further processing steps; e.g., by exposing the polypeptide to enzymes which affect glycosylation, e.g., mammalian glycosylating or deglycosylating enzymes. Also embraced are sequences which have phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine.

 Also included are polypeptides which have been modified using
25 ordinary molecular biological techniques so as to improve their resistance to proteolytic degradation or to optimize solubility properties or to render them more suitable as a therapeutic agent. Analogs of such polypeptides include those containing residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring synthetic amino acids. The peptides of the invention are
30 not limited to products of any of the specific exemplary processes listed herein.

 In another aspect of the present invention, compositions comprising an isolated PKC θ protein may include highly purified truncated version of PKC θ .

As used herein, the term "PKC θ protein of the invention" refers to truncated versions of PKC θ . Substantially pure protein isolated and obtained as described herein may be purified by following known procedures for protein purification, wherein an immunological, enzymatic or other assay is used to monitor purification at each stage in the procedure. Protein purification methods are well known in the art, and are described, for example in Deutscher et al. (ed., 1990, Guide to Protein Purification, Harcourt Brace Jovanovich, San Diego).

Truncated versions of PKC θ useful in the present invention include various deletion mutants of the carboxy-terminal 77 amino acids from PKC θ .

Specifically, the present invention encompasses a PKC θ polypeptide comprising from one to 77 amino acids from the carboxy terminus of a full-length PKC θ polypeptide, wherein the truncated PKC θ polypeptide has the biological activity of interacting with PDK-1. Assays for interaction of PKC θ truncated mutants with PDK-1 are provided elsewhere herein.

Disclosed herein is a method for modulating the activation of PKC θ in a cell. The method of the invention comprises the use of a fragment of PKC θ to modulate the activation of full-length PKC θ by administering a PKC θ fragment to a cell. As described in greater detail herein, an PKC θ fragment of the invention administered to a cell interacts with PDK-1 to prevent the phosphorylation and subsequent activation of PKC θ . In such a method, phosphorylation-mediated activation of PKC θ is detected using a phosphate detection method well known to one of skill in the art. In one aspect of the invention, phosphorylation of PKC θ is detected by probing isolated PKC θ protein with a phospho-specific antibody, as described in the Experimental Details section herein.

"Administration," as used herein, refers to the deliberately-effected presence of an isolated PKC θ protein of the invention or a truncated version thereof.

In one aspect of the invention, isolated truncated PKC θ protein is administered to a cell by incorporating a membrane permeabilizing amino acid sequence on one end of the PKC θ protein fragment. A membrane permeabilizing amino acid sequence can readily be included as part of a PKC θ protein fragment of the invention, for example, by expressing an isolated nucleic acid encoding a fusion protein comprising a nucleic acid sequence encoding a membrane-permeabilizing

sequence located immediately adjacent to a nucleic acid sequence encoding a PKC θ protein fragment of the invention. Such methods of creating fusion proteins are known to one of skill in the art.

5 An isolated truncated PKC θ protein may be administered to a cell by direct expression of the truncated PKC θ protein in the target cell. In one aspect of the invention, an expression vector comprising a PKC θ protein is transfected into a target cell. Expression of the PKC θ protein is effected by means known to one of skill in the art, resulting in in situ production of PKC θ protein.

10 The present invention also presents a method for inhibiting NF- κ B activation in a cell using a PKC θ fragment of the invention by administering a PKC θ fragment to a cell. As described in greater detail herein, an administered PKC θ fragment of the invention interacts with PDK-1 to prevent the phosphorylation and subsequent activation of PKC θ , which in turn prevents NF- κ B activation. In such a method, NF- κ B activation is measured using an NF- κ B reporter plasmid with a
15 luciferase reporter. The design, use, and operation of such plasmids are well known to one of skill in the art.

In one aspect of the invention, a plasmid containing a luciferase reporter gene is transfected into a cell which will subsequently be used to assay the effect of a PKC θ fragment of the invention. A PKC θ fragment of the invention is
20 then administered to the cell and the effect of the PKC θ fragment on NF- κ B activation is evaluated by monitoring luciferase activity.

The present invention also presents a method for modulating T-cell activation using a PKC θ fragment of the invention by administering a PKC θ fragment to a cell. Such a method comprises the use of a fragment of PKC θ to modulate the
25 activation of full-length PKC θ by administering a PKC θ fragment to a cell. As described in greater detail in the Experimental Details section herein, an administered PKC θ fragment of the invention interacts with PDK-1 to prevent the phosphorylation and subsequent activation of PKC θ , which in turn prevents T-cell activation by reducing levels of T-cell receptors such as IL-2 and CD-25-containing high-affinity
30 IL-2. Levels of other T-cell receptors necessary for T-cell activation may also be modulated by administering a PKC θ fragment of the invention. The identity of such T-cell receptors are well known to one of skill in the art.

Inhibition of T-cell receptors is detected in any of a number of ways known to one of skill in the art. In one aspect of the present invention, IL-2 concentration is measured by isolation and direct quantitation of IL-2 levels. In another aspect of the invention, cell surface expression of T-cell receptors CD-25 and CD-69 is assayed and quantified using flow cytometry.

Formation of the supramolecular activation cluster (SMAC) of a T-cell may also be inhibited by administering a PKC θ fragment of the invention according to a method presented by the present invention. As described in greater detail herein, an administered PKC θ fragment of the invention interacts with PDK-1 to prevent the phosphorylation and subsequent activation of PKC θ , which in turn prevents T-cell activation by reducing levels of T-cell receptors such as IL-2 and CD-25-containing high-affinity IL-2, the localization of which is essential for proper formation of the SMAC.

Disclosed herein is a method for modulating the activation of PKC θ in a cell. The method of the invention uses a fragment of PKC θ to modulate the activation of full-length PKC θ by administering a PKC θ fragment to a cell. The present invention also presents a method for modulating T-cell activation using a PKC θ fragment of the invention by administering a PKC θ fragment to a cell. Further, the invention presents a method for suppressing the immune system of an organ transplant patient using a PKC θ fragment of the invention. Formation of the supramolecular activation cluster of a T-cell may also be inhibited by administering a PKC θ fragment of the invention according to a method presented by the present invention.

The present invention includes a method of treating systemic lupus erythematosus (SLE) and organ transplant rejection in a patient by administering a PKC θ fragment to a cell. It will be understood that a patient receiving treatment of any of the above diseases may be an animal, and more preferably a mammal, and even more preferably a human.

The invention also encompasses the use pharmaceutical compositions of an appropriate PKC θ fragment to practice the methods of the invention, the compositions comprising an appropriate PKC θ fragment and a pharmaceutically-acceptable carrier.

As used herein, the term "pharmaceutically-acceptable carrier" means a chemical composition with which an appropriate PKC θ fragment may be combined and which, following the combination, can be used to administer the appropriate PKC θ fragment to a mammal.

5 Pharmaceutical compositions that are useful in the methods of the invention may be administered systemically in oral solid formulations, ophthalmic, suppository, aerosol, topical or other similar formulations. In addition to the appropriate PKC θ fragment, such pharmaceutical compositions may contain pharmaceutically-acceptable carriers and other ingredients known to enhance and
10 facilitate drug administration. Other possible formulations, such as nanoparticles, liposomes, resealed erythrocytes, and immunologically based systems may also be used to administer an appropriate PKC θ fragment according to the methods of the invention.

 Compounds which are identified using any of the methods described
15 herein may be formulated and administered to a mammal for treatment of the diseases disclosed herein are now described.

 The invention encompasses the preparation and use of pharmaceutical compositions comprising a compound useful for treatment of the diseases disclosed herein as an active ingredient. Such a pharmaceutical composition may consist of the
20 active ingredient alone, in a form suitable for administration to a subject, or the pharmaceutical composition may comprise the active ingredient and one or more pharmaceutically acceptable carriers, one or more additional ingredients, or some combination of these. The active ingredient may be present in the pharmaceutical composition in the form of a physiologically acceptable ester or salt, such as in
25 combination with a physiologically acceptable cation or anion, as is well known in the art.

 As used herein, the term "physiologically acceptable" ester or salt means an ester or salt form of the active ingredient which is compatible with any other ingredients of the pharmaceutical composition, which is not deleterious to the
30 subject to which the composition is to be administered.

 The formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the

active ingredient into association with a carrier or one or more other accessory ingredients, and then, if necessary or desirable, shaping or packaging the product into a desired single- or multi-dose unit.

Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for ethical administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to animals of all sorts.

Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and perform such modification with merely ordinary, if any, experimentation. Subjects to which administration of the pharmaceutical compositions of the invention is contemplated include, but are not limited to, humans and other primates, mammals including commercially relevant mammals such as cattle, pigs, horses, sheep, cats, and dogs, birds including commercially relevant birds such as chickens, ducks, geese, and turkeys.

Pharmaceutical compositions that are useful in the methods of the invention may be prepared, packaged, or sold in formulations suitable for oral, rectal, vaginal, parenteral, topical, pulmonary, intranasal, buccal, ophthalmic, intrathecal or another route of administration. Other contemplated formulations include projected nanoparticles, liposomal preparations, resealed erythrocytes containing the active ingredient, and immunologically-based formulations.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in bulk, as a single unit dose, or as a plurality of single unit doses. As used herein, a "unit dose" is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

The relative amounts of the active ingredient, the pharmaceutically acceptable carrier, and any additional ingredients in a pharmaceutical composition of the invention will vary, depending upon the identity, size, and condition of the subject treated and further depending upon the route by which the composition is to be

administered. By way of example, the composition may comprise between 0.1% and 100% (w/w) active ingredient.

5 In addition to the active ingredient, a pharmaceutical composition of the invention may further comprise one or more additional pharmaceutically active agents. Particularly contemplated additional agents include anti-emetics and scavengers such as cyanide and cyanate scavengers.

Controlled- or sustained-release formulations of a pharmaceutical composition of the invention may be made using conventional technology.

10 A formulation of a pharmaceutical composition of the invention suitable for oral administration may be prepared, packaged, or sold in the form of a discrete solid dose unit including, but not limited to, a tablet, a hard or soft capsule, a cachet, a troche, or a lozenge, each containing a predetermined amount of the active ingredient. Other formulations suitable for oral administration include, but are not limited to, a powdered or granular formulation, an aqueous or oily suspension, an aqueous or oily solution, or an emulsion.

As used herein, an "oily" liquid is one which comprises a carbon-containing liquid molecule and which exhibits a less polar character than water.

20 A tablet comprising the active ingredient may, for example, be made by compressing or molding the active ingredient, optionally with one or more additional ingredients. Compressed tablets may be prepared by compressing, in a suitable device, the active ingredient in a free-flowing form such as a powder or granular preparation, optionally mixed with one or more of a binder, a lubricant, an excipient, a surface active agent, and a dispersing agent. Molded tablets may be made by molding, in a suitable device, a mixture of the active ingredient, a pharmaceutically acceptable carrier, and at least sufficient liquid to moisten the mixture. Pharmaceutically acceptable excipients used in the manufacture of tablets include, but are not limited to, inert diluents, granulating and disintegrating agents, binding agents, and lubricating agents. Known dispersing agents include, but are not limited to, potato starch and sodium starch glycollate. Known surface active agents include, but are not limited to, sodium lauryl sulphate. Known diluents include, but are not limited to, calcium carbonate, sodium carbonate, lactose, microcrystalline cellulose, calcium phosphate, calcium hydrogen phosphate, and sodium phosphate. Known granulating and disintegrating agents include, but are not limited to, corn

starch and alginic acid. Known binding agents include, but are not limited to, gelatin, acacia, pre-gelatinized maize starch, polyvinylpyrrolidone, and hydroxypropyl methylcellulose. Known lubricating agents include, but are not limited to, magnesium stearate, stearic acid, silica, and talc.

5 Tablets may be non-coated or they may be coated using known methods to achieve delayed disintegration in the gastrointestinal tract of a subject, thereby providing sustained release and absorption of the active ingredient. By way of example, a material such as glyceryl monostearate or glyceryl distearate may be used to coat tablets. Further by way of example, tablets may be coated using methods
10 described in U.S. Patents numbers 4,256,108; 4,160,452; and 4,265,874 to form osmotically-controlled release tablets. Tablets may further comprise a sweetening agent, a flavoring agent, a coloring agent, a preservative, or some combination of these in order to provide pharmaceutically elegant and palatable preparation.

 Hard capsules comprising the active ingredient may be made using a
15 physiologically degradable composition, such as gelatin. Such hard capsules comprise the active ingredient, and may further comprise additional ingredients including, for example, an inert solid diluent such as calcium carbonate, calcium phosphate, or kaolin.

 Soft gelatin capsules comprising the active ingredient may be made
20 using a physiologically degradable composition, such as gelatin. Such soft capsules comprise the active ingredient, which may be mixed with water or an oil medium such as peanut oil, liquid paraffin, or olive oil.

 Liquid formulations of a pharmaceutical composition of the invention which are suitable for oral administration may be prepared, packaged, and sold either
25 in liquid form or in the form of a dry product intended for reconstitution with water or another suitable vehicle prior to use.

 Liquid suspensions may be prepared using conventional methods to achieve suspension of the active ingredient in an aqueous or oily vehicle. Aqueous vehicles include, for example, water and isotonic saline. Oily vehicles include, for
30 example, almond oil, oily esters, ethyl alcohol, vegetable oils such as arachis, olive, sesame, or coconut oil, fractionated vegetable oils, and mineral oils such as liquid paraffin. Liquid suspensions may further comprise one or more additional ingredients including, but not limited to, suspending agents, dispersing or wetting agents,

emulsifying agents, demulcents, preservatives, buffers, salts, flavorings, coloring agents, and sweetening agents. Oily suspensions may further comprise a thickening agent. Known suspending agents include, but are not limited to, sorbitol syrup, hydrogenated edible fats, sodium alginate, polyvinylpyrrolidone, gum tragacanth, gum acacia, and cellulose derivatives such as sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose. Known dispersing or wetting agents include, but are not limited to, naturally-occurring phosphatides such as lecithin, condensation products of an alkylene oxide with a fatty acid, with a long chain aliphatic alcohol, with a partial ester derived from a fatty acid and a hexitol, or with a partial ester derived from a fatty acid and a hexitol anhydride (e.g. polyoxyethylene stearate, heptadecaethyleneoxycetanol, polyoxyethylene sorbitol monooleate, and polyoxyethylene sorbitan monooleate, respectively). Known emulsifying agents include, but are not limited to, lecithin and acacia. Known preservatives include, but are not limited to, methyl, ethyl, or n-propyl-para- hydroxybenzoates, ascorbic acid, and sorbic acid. Known sweetening agents include, for example, glycerol, propylene glycol, sorbitol, sucrose, and saccharin. Known thickening agents for oily suspensions include, for example, beeswax, hard paraffin, and cetyl alcohol.

Liquid solutions of the active ingredient in aqueous or oily solvents may be prepared in substantially the same manner as liquid suspensions, the primary difference being that the active ingredient is dissolved, rather than suspended in the solvent. Liquid solutions of the pharmaceutical composition of the invention may comprise each of the components described with regard to liquid suspensions, it being understood that suspending agents will not necessarily aid dissolution of the active ingredient in the solvent. Aqueous solvents include, for example, water and isotonic saline. Oily solvents include, for example, almond oil, oily esters, ethyl alcohol, vegetable oils such as arachis, olive, sesame, or coconut oil, fractionated vegetable oils, and mineral oils such as liquid paraffin.

Powdered and granular formulations of a pharmaceutical preparation of the invention may be prepared using known methods. Such formulations may be administered directly to a subject, used, for example, to form tablets, to fill capsules, or to prepare an aqueous or oily suspension or solution by addition of an aqueous or oily vehicle thereto. Each of these formulations may further comprise one or more of dispersing or wetting agent, a suspending agent, and a preservative. Additional

excipients, such as fillers and sweetening, flavoring, or coloring agents, may also be included in these formulations.

A pharmaceutical composition of the invention may also be prepared, packaged, or sold in the form of oil-in-water emulsion or a water-in-oil emulsion.

5 The oily phase may be a vegetable oil such as olive or arachis oil, a mineral oil such as liquid paraffin, or a combination of these. Such compositions may further comprise one or more emulsifying agents such as naturally occurring gums such as gum acacia or gum tragacanth, naturally-occurring phosphatides such as soybean or lecithin phosphatide, esters or partial esters derived from combinations of fatty acids
10 and hexitol anhydrides such as sorbitan monooleate, and condensation products of such partial esters with ethylene oxide such as polyoxyethylene sorbitan monooleate. These emulsions may also contain additional ingredients including, for example, sweetening or flavoring agents.

A pharmaceutical composition of the invention may be prepared,
15 packaged, or sold in a formulation suitable for rectal administration. Such a composition may be in the form of, for example, a suppository, a retention enema preparation, and a solution for rectal or colonic irrigation.

Suppository formulations may be made by combining the active ingredient with a non-irritating pharmaceutically acceptable excipient which is solid
20 at ordinary room temperature (i.e. about 20 °C) and which is liquid at the rectal temperature of the subject (i.e. about 37 °C in a healthy human). Suitable pharmaceutically acceptable excipients include, but are not limited to, cocoa butter, polyethylene glycols, and various glycerides. Suppository formulations may further comprise various additional ingredients including, but not limited to, antioxidants and
25 preservatives.

Retention enema preparations or solutions for rectal or colonic irrigation may be made by combining the active ingredient with a pharmaceutically acceptable liquid carrier. As is well known in the art, enema preparations may be administered using, and may be packaged within, a delivery device adapted to the
30 rectal anatomy of the subject. Enema preparations may further comprise various additional ingredients including, but not limited to, antioxidants and preservatives.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for vaginal administration. Such a

composition may be in the form of, for example, a suppository, an impregnated or coated vaginally-insertable material such as a tampon, a douche preparation, or gel or cream or a solution for vaginal irrigation.

5 Methods for impregnating or coating a material with a chemical composition are known in the art, and include, but are not limited to methods of depositing or binding a chemical composition onto a surface, methods of incorporating a chemical composition into the structure of a material during the synthesis of the material (i.e. such as with a physiologically degradable material), and methods of absorbing an aqueous or oily solution or suspension into an absorbent material, with or without subsequent drying.

 Douche preparations or solutions for vaginal irrigation may be made by combining the active ingredient with a pharmaceutically acceptable liquid carrier. As is well known in the art, douche preparations may be administered using, and may be packaged within, a delivery device adapted to the vaginal anatomy of the subject.

15 Douche preparations may further comprise various additional ingredients including, but not limited to, antioxidants, antibiotics, antifungal agents, and preservatives.

 As used herein, "parenteral administration" of a pharmaceutical composition includes any route of administration characterized by physical breaching of a tissue of a subject and administration of the pharmaceutical composition through the breach in the tissue. Parenteral administration thus includes, but is not limited to, administration of a pharmaceutical composition by injection of the composition, by application of the composition through a surgical incision, by application of the composition through a tissue-penetrating non-surgical wound, and the like. In particular, parenteral administration is contemplated to include, but is not limited to,

20 subcutaneous, intraperitoneal, intramuscular, intrasternal injection, and kidney dialytic infusion techniques.

 Formulations of a pharmaceutical composition suitable for parenteral administration comprise the active ingredient combined with a pharmaceutically acceptable carrier, such as sterile water or sterile isotonic saline. Such formulations may be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable formulations may be prepared, packaged, or sold in unit dosage form, such as in ampules or in multi-dose containers containing a preservative. Formulations for parenteral administration include, but are not limited

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to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable sustained-release or biodegradable formulations. Such formulations may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing, or dispersing agents. In one embodiment of a formulation for
5 parenteral administration, the active ingredient is provided in dry (i.e. powder or granular) form for reconstitution with a suitable vehicle (e.g. sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition.

The pharmaceutical compositions may be prepared, packaged, or sold in the form of a sterile injectable aqueous or oily suspension or solution. This
10 suspension or solution may be formulated according to the known art, and may comprise, in addition to the active ingredient, additional ingredients such as the dispersing agents, wetting agents, or suspending agents described herein. Such sterile injectable formulations may be prepared using a non-toxic parenterally-acceptable diluent or solvent, such as water or 1,3-butane diol, for example. Other acceptable
15 diluents and solvents include, but are not limited to, Ringer's solution, isotonic sodium chloride solution, and fixed oils such as synthetic mono- or di-glycerides. Other parentally-administrable formulations which are useful include those which comprise the active ingredient in microcrystalline form, in a liposomal preparation, or as a component of a biodegradable polymer systems. Compositions for sustained release
20 or implantation may comprise pharmaceutically acceptable polymeric or hydrophobic materials such as an emulsion, an ion exchange resin, a sparingly soluble polymer, or a sparingly soluble salt.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for pulmonary administration via the
25 buccal cavity. Such a formulation may comprise dry particles which comprise the active ingredient and which have a diameter in the range from about 0.5 to about 7 nanometers, and preferably from about 1 to about 6 nanometers. Such compositions are conveniently in the form of dry powders for administration using a device comprising a dry powder reservoir to which a stream of propellant may be directed to
30 disperse the powder or using a self-propelling solvent/powder-dispensing container such as a device comprising the active ingredient dissolved or suspended in a low-boiling propellant in a sealed container. Preferably, such powders comprise particles wherein at least 98% of the particles by weight have a diameter greater than 0.5

nanometers and at least 95% of the particles by number have a diameter less than 7 nanometers. More preferably, at least 95% of the particles by weight have a diameter greater than 1 nanometer and at least 90% of the particles by number have a diameter less than 6 nanometers. Dry powder compositions preferably include a solid fine powder diluent such as sugar and are conveniently provided in a unit dose form.

Low boiling propellants generally include liquid propellants having a boiling point of below 65 °F at atmospheric pressure. Generally the propellant may constitute 50 to 99.9% (w/w) of the composition, and the active ingredient may constitute 0.1 to 20% (w/w) of the composition. The propellant may further comprise additional ingredients such as a liquid non-ionic or solid anionic surfactant or a solid diluent (preferably having a particle size of the same order as particles comprising the active ingredient).

Pharmaceutical compositions of the invention formulated for pulmonary delivery may also provide the active ingredient in the form of droplets of a solution or suspension. Such formulations may be prepared, packaged, or sold as aqueous or dilute alcoholic solutions or suspensions, optionally sterile, comprising the active ingredient, and may conveniently be administered using any nebulization or atomization device. Such formulations may further comprise one or more additional ingredients including, but not limited to, a flavoring agent such as saccharin sodium, a volatile oil, a buffering agent, a surface active agent, or a preservative such as methylhydroxybenzoate. The droplets provided by this route of administration preferably have an average diameter in the range from about 0.1 to about 200 nanometers.

The formulations described herein as being useful for pulmonary delivery are also useful for intranasal delivery of a pharmaceutical composition of the invention.

Another formulation suitable for intranasal administration is a coarse powder comprising the active ingredient and having an average particle from about 0.2 to 500 micrometers. Such a formulation is administered in the manner in which snuff is taken i.e. by rapid inhalation through the nasal passage from a container of the powder held close to the nares.

Formulations suitable for nasal administration may, for example, comprise from about as little as 0.1% (w/w) and as much as 100% (w/w) of the active

ingredient, and may further comprise one or more of the additional ingredients described herein.

5 A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for buccal administration. Such formulations may, for example, be in the form of tablets or lozenges made using conventional methods, and may, for example, 0.1 to 20% (w/w) active ingredient, the balance comprising an orally dissolvable or degradable composition and, optionally, one or more of the additional ingredients described herein. Alternately, formulations suitable for buccal administration may comprise a powder or an aerosolized or
10 atomized solution or suspension comprising the active ingredient. Such powdered, aerosolized, or aerosolized formulations, when dispersed, preferably have an average particle or droplet size in the range from about 0.1 to about 200 nanometers, and may further comprise one or more of the additional ingredients described herein.

A pharmaceutical composition of the invention may be prepared,
15 packaged, or sold in a formulation suitable for ophthalmic administration. Such formulations may, for example, be in the form of eye drops including, for example, a 0.1-1.0% (w/w) solution or suspension of the active ingredient in an aqueous or oily liquid carrier. Such drops may further comprise buffering agents, salts, or one or more other of the additional ingredients described herein. Other ophthalmically-
20 administrable formulations which are useful include those which comprise the active ingredient in microcrystalline form or in a liposomal preparation.

As used herein, "additional ingredients" include, but are not limited to, one or more of the following: excipients; surface active agents; dispersing agents; inert diluents; granulating and disintegrating agents; binding agents; lubricating
25 agents; sweetening agents; flavoring agents; coloring agents; preservatives; physiologically degradable compositions such as gelatin; aqueous vehicles and solvents; oily vehicles and solvents; suspending agents; dispersing or wetting agents; emulsifying agents, demulcents; buffers; salts; thickening agents; fillers; emulsifying agents; antioxidants; antibiotics; antifungal agents; stabilizing agents; and
30 pharmaceutically acceptable polymeric or hydrophobic materials. Other "additional ingredients" which may be included in the pharmaceutical compositions of the invention are known in the art and described, for example in Genaro, ed., 1985,

Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, which is incorporated herein by reference.

Typically dosages of the compound of the invention which may be administered to an animal, preferably a human, range in amount from 1 g to about 100 g per killogram of body weight of the animal. While the precise dosage administered will vary depending upon any number of factors, including but not limited to, the type of animal and type of disease state being treated, the age of the animal and the route of administration. Preferably, the dosage of the compound will vary from about 1 mg to about 10 g per killogram of body weight of the animal. More preferably, the dosage will vary from about 10 mg to about 1 g per killogram of body weight of the animal.

The compound may be administered to an animal as frequently as several times daily, or it may be administered less frequently, such as once a day, once a week, once every two weeks, once a month, or even less frequently, such as once every several months or even once a year or less. The frequency of the dose will be readily apparent to the skilled artisan and will depend upon any number of factors, such as, but not limited to, the type and severity of the disease being treated, the type and age of the animal, etc.

Definitions

The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

5 "Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom.

10 Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the

15 protein or other product of that gene or cDNA.

 An "isolated nucleic acid" refers to a nucleic acid segment or fragment which has been separated from sequences which flank it in a naturally occurring state, e.g., a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment, e.g., the sequences adjacent to the fragment in a

20 genome in which it naturally occurs. The term also applies to nucleic acids which have been substantially purified from other components which naturally accompany the nucleic acid, e.g., RNA or DNA or proteins, which naturally accompany it in the cell. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into

25 the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., as a cDNA or a genomic or cDNA fragment produced by PCR or restriction enzyme digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

30 In the context of the present invention, the following abbreviations for the commonly occurring nucleic acid bases are used. "A" refers to adenosine, "C" refers to cytidine, "G" refers to guanosine, "T" refers to thymidine, and "U" refers to uridine.

A "polynucleotide" means a single strand or parallel and anti-parallel strands of a nucleic acid. Thus, a polynucleotide may be either a single-stranded or a double-stranded nucleic acid.

The term "nucleic acid" typically refers to large polynucleotides.

5 The term "oligonucleotide" typically refers to short polynucleotides, generally no greater than about 50 nucleotides. It will be understood that when a nucleotide sequence is represented by a DNA sequence (i.e., A, T, G, C), this also includes an RNA sequence (i.e., A, U, G, C) in which "U" replaces "T."

10 Conventional notation is used herein to describe polynucleotide sequences: the left-hand end of a single-stranded polynucleotide sequence is the 5'-end; the left-hand direction of a double-stranded polynucleotide sequence is referred to as the 5'-direction.

15 The direction of 5' to 3' addition of nucleotides to nascent RNA transcripts is referred to as the transcription direction. The DNA strand having the same sequence as an mRNA is referred to as the "coding strand"; sequences on the DNA strand which are located 5' to a reference point on the DNA are referred to as "upstream sequences"; sequences on the DNA strand which are 3' to a reference point on the DNA are referred to as "downstream sequences."

20 Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

25 "Homologous" as used herein, refers to nucleotide sequence similarity between two regions of the same nucleic acid strand or between regions of two different nucleic acid strands. When a nucleotide residue position in both regions is occupied by the same nucleotide residue, then the regions are homologous at that position. A first region is homologous to a second region if at least one nucleotide residue position of each region is occupied by the same residue. Homology between two regions is expressed in terms of the proportion of nucleotide residue positions of the two regions that are occupied by the same nucleotide residue. By way of
30 example, a region having the nucleotide sequence 5'-ATTGCC-3' and a region having the nucleotide sequence 5'-TATGGC-3' share 50% homology. Preferably, the first region comprises a first portion and the second region comprises a second portion,

whereby, at least about 50%, and preferably at least about 75%, at least about 90%, or at least about 95% of the nucleotide residue positions of each of the portions are occupied by the same nucleotide residue. More preferably, all nucleotide residue positions of each of the portions are occupied by the same nucleotide residue.

5 As used herein, "homology" is used synonymously with "identity."

"Polypeptide" refers to a polymer composed of amino acid residues, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof linked via peptide bonds, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof. Synthetic
10 polypeptides can be synthesized, for example, using an automated polypeptide synthesizer.

The term "protein" typically refers to large polypeptides.

The term "peptide" typically refers to short polypeptides. A peptide "fragment" is typically a peptide comprising a linear portion of a total protein
15 sequence.

Conventional notation is used herein to portray polypeptide sequences: the left-hand end of a polypeptide sequence is the amino-terminus; the right-hand end of a polypeptide sequence is the carboxyl-terminus.

The term "fusion protein" or "fusion peptide" refers to a protein or
20 peptide, respectively, that has an additional protein or peptide sequence attached via peptide bonding to the amino terminus or carboxy terminus. Alternatively, a fusion protein or fusion peptide may have more than one fusion polypeptide sequence attached at the amino terminus, carboxy terminus, or both. Furthermore, a fusion polypeptide may consist of two or more polypeptides connected via peptide bonding
25 in series. Such fusion proteins or fusion peptides may be produced by translation of a polynucleotide or by in vitro protein chemistry methods known in the art.

A first defined nucleic acid sequence is said to be "immediately adjacent to" a second defined nucleic acid sequence when, for example, the last
30 nucleotide of the first nucleic acid sequence is chemically bonded to the first nucleotide of the second nucleic acid sequence through a phosphodiester bond. Conversely, a first defined nucleic acid sequence is also said to be "immediately adjacent to" a second defined nucleic acid sequence when, for example, the first nucleotide of the first nucleic acid sequence is chemically bonded to the last

nucleotide of the second nucleic acid sequence through a phosphodiester bond. By way of example, the pentanucleotide 5'-AAAAA-3' is adjacent the trinucleotide 5'-TTT-3' when the two are connected thus: 5'-AAAAATTT-3' or 5'-TTTAAAAA-3', but not when the two are connected thus: 5'-AAAAACTTT-3'.

5 A first defined polypeptide sequence is said to be "immediately adjacent to" a second defined polypeptide sequence when, for example, the last amino acid of the first polypeptide sequence is chemically bonded to the first amino acid of the second polypeptide sequence through a peptide bond. Conversely, a first defined polypeptide sequence is said to be "immediately adjacent to" a second defined
10 polypeptide sequence when, for example, the first amino acid of the first polypeptide sequence is chemically bonded to the last amino acid of the second polypeptide sequence through a peptide bond.

 A "membrane-permeabilizing sequence" refers to a peptide or protein that has the native ability to cross a cell membrane. Additionally, when a membrane-
15 permeabilizing sequence comprises a fusion protein with another polypeptide, the membrane-permeabilizing sequence will facilitate the transmembrane transport of the entire fusion protein.

 "Modulation of activity of a polypeptide" refers to an increase or a decrease in the rate or degree of biological activity of the polypeptide, an increase or a
20 decrease in the affinity of the polypeptide for a target, an increase or a decrease in the expression levels of the polypeptide, or an increase or a decrease in the biological effect mediated by the polypeptide. "Modulation" as it is used herein refers to any change from the current condition or status of a molecule. For example, modulation of a protein can be the activation, down-regulation, inhibition, or change in
25 conformation of the protein.

 A "vector" is a composition of matter which comprises an isolated nucleic acid and which can be used to deliver the isolated nucleic acid to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds,
30 plasmids, and viruses. Thus, the term "vector" includes an autonomously replicating plasmid or a virus. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the like. Examples of viral vectors

include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, and the like.

“Expression vector” refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an in vitro expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (e.g., naked or contained in liposomes) and viruses that incorporate the recombinant polynucleotide.

A “disease” is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal's health continues to deteriorate. In contrast, a “disorder” in an animal is a state of health in which the animal is able to maintain homeostasis, but in which the animal's state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal's state of health.

A disease or disorder is “alleviated” if the severity of a symptom of the disease or disorder, the frequency with which such a symptom is experienced by a patient, or both, are reduced.

As used herein, a “functional” biological molecule is a biological molecule in a form in which it exhibits a property by which it is characterized. A functional enzyme, for example, is one which exhibits the characteristic catalytic activity by which the enzyme is characterized.

A “ligand” is a compound that specifically binds to a target receptor.

A “receptor” is a compound that specifically binds to a ligand.

A ligand or a receptor (e.g., an antibody) “specifically binds to” or “is specifically immunoreactive with” a compound when the ligand or receptor functions in a binding reaction which is determinative of the presence of the compound in a sample of heterogeneous compounds. Thus, under designated assay (e.g., immunoassay) conditions, the ligand or receptor binds preferentially to a particular compound and does not bind in a significant amount to other compounds present in the sample. For example, a polynucleotide specifically binds under hybridization conditions to an compound polynucleotide comprising a complementary sequence; an

antibody specifically binds under immunoassay conditions to an antigen bearing an epitope against which the antibody was raised. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select
5 monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane (1988, Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, New York) for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

Two proteins that specifically bind to each other can be said to
10 “selectively interact.”

“Primer” refers to a polynucleotide that is capable of specifically hybridizing to a designated polynucleotide template and providing a point of initiation for synthesis of a complementary polynucleotide. Such synthesis occurs when the polynucleotide primer is placed under conditions in which synthesis is induced, i.e., in
15 the presence of nucleotides, a complementary polynucleotide template, and an agent for polymerization such as DNA polymerase. A primer is typically single-stranded, but may be double-stranded. Primers are typically deoxyribonucleic acids, but a wide variety of synthetic and naturally occurring primers are useful for many applications. A primer is complementary to the template to which it is designed to hybridize to
20 serve as a site for the initiation of synthesis, but need not reflect the exact sequence of the template. In such a case, specific hybridization of the primer to the template depends on the stringency of the hybridization conditions. Primers can be labeled with, e.g., chromogenic, radioactive, or fluorescent moieties and used as detectable moieties.

25 As used herein, “alleviating a symptom” means reducing the severity of the symptom.

As used herein, “treating a disorder” means reducing the frequency with which a symptom of the disorder is experienced by a patient.

A “prophylactic” treatment is a treatment administered to a subject
30 who does not exhibit signs of a disease or exhibits only early signs of the disease for the purpose of decreasing the risk of developing pathology associated with the disease.

A "therapeutic" treatment is a treatment administered to a subject who exhibits signs of pathology for the purpose of diminishing or eliminating those signs.

5 A "therapeutically effective amount" of a compound is that amount of compound which is sufficient to provide a beneficial effect to the subject to which the compound is administered.

As used herein, the terms "administer" or "administration," refers to the deliberately-effected presence of an isolated PKC θ protein of the invention or a truncated version thereof. By way of example, a PKC θ protein fragment may be administered to a cell by creating a fusion protein comprising the PKC θ protein
10 fragment and a membrane permeabilizing sequence and contacting the cell with the fusion protein such that the fusion protein is transported across the cell membrane and into the cell.

As used herein, an "instructional material" includes a publication, a recording, a diagram, or any other medium of expression which can be used to
15 communicate the usefulness of the composition of the invention for its designated use. The instructional material of the kit of the invention may, for example, be affixed to a container which contains the composition or be shipped together with a container which contains the composition. Alternatively, the instructional material may be shipped separately from the container with the intention that the instructional material
20 and the composition be used cooperatively by the recipient.

EXPERIMENTAL EXAMPLES

The invention is now described with reference to the following examples. These examples are provided for the purpose of illustration only and the
25 invention should in no way be construed as being limited to these examples but rather should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Analysis of PKC θ was first conducted by co-expressing in cells a
30 dominant-negative PI3-kinase mutant (p 85) along with PKC θ and measured NF- κ B activation using a NF- κ B reporter plasmid. Overexpression of a constitutively active (ca) mutant of PKC θ (PKC θ A148E) that was mutated in the pseudosubstrate site and

was shown to be functionally independent of any agonist, induced a 5 to 10 fold increase of NF- κ B activation. A dominant-negative PI3-kinase mutant (p 85) exhibited no effect if transfected alone but when co-expressed with PKC θ completely blocked NF- κ B activation (Fig. 1B). Consistent with these results, stimulation of primary T cells from mice transgenically expressing a NF- κ B-dependent luciferase reporter (Voll et al., 2000, Immunity 13:677-89), with anti CD3/CD28, induced a marked increase of luciferase activity while pre-incubation with wortmannin or LY92024, two well-characterized PI3-kinase inhibitors, inhibited luciferase expression (Fig. 1C).

To determine if PKC θ -induced NF- κ B activation was also regulated by PDK-1, PKC θ was co-transfected with PDK-1 into cells. Transfection of cells with PKC θ ca caused significant activation of NF- κ B while PKC θ wild-type (PKC θ wt) exhibited only a modest increase. A PKC θ kinase inactive (mut) mutant (PKC θ K409R), strongly reduced NF- κ B activation. Co-transfection of PKC θ wt or PKC θ ca with PDK-1 increased luciferase activity by about 10 and 30 fold, respectively, whereas PKC θ mut showed no effect (Fig. 1D). To further confirm that PDK-1 is upstream of PKC θ , PKC θ was co-transfected with either a constitutively active (A280V) mutant of PDK-1 that has been shown to bypass the requirement of PI3-kinase (Paradis et al., 1999, Genes Dev 13:1438-52) or a kinase dead mutant of (K111A/D223A) (Alessi, et al., 1977, Curr Biol 7:776-89) PDK-1. Coexpression of wild type or constitutively active PDK-1 significantly increased PKC θ -induced NF- κ B activation while the kinase-dead mutant failed to lead to NF- κ B activation, thus suggesting that the phosphorylation of PKC θ by PDK-1 is an essential step for the subsequent activation of NF- κ B (Fig. 1E).

Recent studies have shown that PDK-1 regulates PKCs by phosphorylating the activation loop and a hydrophobic motif in the COOH-terminal end (Le Good et al., 1998, Science 281:2042-5; Gao et al., 2001, J. Biol. Chem. 276:19588-96; Balendran et al., FEBS Lett. 484:217-23). Sequence alignment analysis of members of conventional (PKC II), novel (PKC δ) and atypical (PKC ζ) subclass (Fig. 1F) demonstrated that PKC θ also contained a conserved threonine residue in the activation loop (threonine 538) and a serine residue in the hydrophobic motif (serine 695) (Fig. 1G). To determine whether these two residues are actually

phosphorylated during T cell activation, immunoblotting experiments were carried out with a phospho-specific antiserum that specifically recognizes PKC θ phosphorylated on threonine 538, and a phospho-PKC (pan) antibody that detects different PKC isoforms only when phosphorylated at the carboxy-terminal serine residue.

5 Stimulation of primary T cells or Jurkat cells with anti-CD3/CD28 caused phosphorylation of Thr538 within 60 minutes. The immunoblotting results with the phospho-PKC (pan) antibody demonstrated that Ser695 is constitutively phosphorylated in resting cells. Engagement of TCR with anti-CD3/CD2S caused the dephosphorylation of this residue followed by the re-phosphorylation within 60 min
10 suggesting a more complex regulation of this residue probably through the involvement of a phosphatase as already suggested for other protein kinases (Balendran, et al., 1999, Curr Biol 9:393-404) (Fig. 1H). Pretreatment of T-cells with wortmanin or LY924024 inhibited the PDK-1 mediated phosphorylation of both Thr538 and Ser695. Finally, transfection into cells of mutants of PKC θ with
15 Thr538Ala or Ser695Ala showed significantly reduced NF- κ B activation either when transfected alone or together with PDK-1 (Fig. 1I). Taken together, these findings suggest that PDK-1 is the upstream kinase of PKC θ , and that phosphorylation of Thr 538 and Ser 695 by PDK-1 are important steps for PKC θ -activation upon TCR-stimulation in T-cells.

20 To assess the interaction of PDK-1 and PKC θ , the proteins were co-expressed in HeLa cells. Immunoprecipitation of PDK-1 followed by immunoblotting demonstrated that PKC θ could associate with PDK-1 (Fig. 2A, lane 1). Conversely, PDK-1 could be immunoprecipitated with PKC θ . Complex formation between PKCs and PDK-1 has been shown to require a hydrophobic motif Phe-X-X-Phe-Ser/Thr-
25 Phe/Tyr, where X can be any amino acid, in the COOH-terminus of PKCs (Balendran, 1999, J Biol Chem 274:37400-6). Analysis of sequence alignments of different members of PKC family with PKC θ reveals the presence of a similar motif in PKC θ , and so the role of this region in the interaction with PDK-1 and its effect on NF- κ B activation was examined. Deletions within the carboxyl terminal domain (1-622,
30 1665, 1-684) of PKC θ (Fig. 2A) revealed that the hydrophobic motif of this protein was essential for complex formation with PDK-1 (Fig. 2B). To assess the possible functional consequence of this interaction, the same mutants were co-transfected

along with PDK-1 and measured NF- κ B activation. PKC θ deletion mutants showed a slight decrease in NF- κ B activation when transfected alone as compared to full length PKC θ , however co-transfection with PDK-1 led to a marked decrease of NF- κ B activation by the truncated mutants as compared to full length PKC θ (Fig. 2C).

5 Therefore these observations lead to the conclusion that PDK-1 interacts primarily with determinants residing in the COOH-terminal hydrophobic motif of PKC θ and that the lack of PDK-1 binding domain in PKC θ severely affects its ability to activate NF- κ B.

To further confirm that this interaction plays a key role in PKC θ signaling to NF- κ B, a construct was generated containing the COOH-terminal domain of PKC θ (a.a. 622-706) as a GST fusion protein that we have named GST-PIF θ (Glutathione-S-transferase fusion with EDK-1 interacting fragment of PKC θ) and tested the effect of its co-expression on NF- κ B activation or P1CC 8 co-immunoprecipitation with PDK-1. As shown in Fig. 2D co-transfection into cells of increasing amount of GST-PIF θ along with PKC θ and PDK-1 inhibits PKC θ induced NF- κ B activation in a dose dependent manner whereas transfection of GST vector alone had no effect. Similarly co-transfection into 293 cells of GST-PIF θ together with PKC θ and PDK-1 dramatically reduced the PKC θ -PDK-1 interaction (Fig. 2 E) confirming once again that blocking the interaction of PKC θ with PDK-1 leads to a marked decrease in PIF θ -induced NF- κ B activation. Finally, cotransfection of GST-PIF θ with PDK-1 and immunoprecipitation with glutathione sepharose beads confirmed that GST-PIF θ efficiently binds PDK-1 (Fig. 2 F). Consistent with these findings several studies have recently demonstrated that over-expression of a region of protein kinase C-related kinase-2 (PRK2), termed PIF, has the ability to complex with PDK-1 and block the activation and phosphorylation of p70S6K (May, et al., 2000, Science 289:1550-4).

To identify the domain of PDK-1 that interacts with COOH terminal domain of PKC θ , three HA-tagged deletion mutants of PDK-1 were generated. The deletion mutants contained the NH2-terminus, the catalytic domain and the pleckstrin homology domain (Fig. 2 G) and were tested in a co-immunoprecipitation assay with GST-PIF θ . As shown in Fig. 2G, GST-PIF θ interacted with the construct expressing

the PDK-1 catalytic domain (PDK-1 84-332) (Fig. 2 G, right panel, lane 2) but not with PDK-1 1-84 or 341-559 constructs (data not shown) suggesting that the catalytic domain of PDK-1 is necessary for the interaction with the carboxyl terminal domain of PKC θ . Moreover, when the PDK-1 deletion mutants were co-expressed with PKC θ ca
5 in Hela cells to measure the activation of NF- κ B, only the full-length PDK-1 and mutant containing the catalytic domain (84-341) was able to increase PKC θ -induced NF- κ B activation (Fig. 2G).

Given the critical role of the PIF domain in PKC θ -induced NF- κ B activation, the importance of this interaction in the context of T cell activation was
10 evaluated. A cell permeable peptide spanning the PKC θ PIF domain was designed, and its ability to block the interaction between PKC θ and PDK-1 in T cells was tested. The wild type PIF θ peptide contained sequences from methionine 686 to proline 699 of PKC θ fused to a membrane-permeabilizing sequence derived from the *Antennapedia* homeodomain protein. As a control, a randomized version of the PIF-9
15 peptide was fused with the Antennapedia sequence (Fig. 3A). When added to T-cells, only the wild type PIF θ peptide inhibited the *in vivo* interaction of PKC θ with PDK-1 in a concentration-dependent manner (Fig. 3B, lanes 3 and 4). Electrophoretic mobility shift assays (EMSA) demonstrated that only the wild type but not the scrambled peptide inhibited anti-CD3/CD28 stimulated NF- κ B activation in Jurkat
20 cells. In contrast, neither peptide affected TNF- α induced NF- κ B activation demonstrating the specificity of the PDK-1 –PKC θ -NF- κ B pathway for only the TCR-induced pathways (Fig. 3C). Furthermore, the wild type, but not the scrambled PIF-peptide, inhibited TCR-induced luciferase activity in primary T-cells isolated from KB-luciferase transgenic mice. (Fig. 3D). Once again, the peptides had no effect on
25 TNF α -stimulated NF- κ B in these primary T-cells (Fig. 3D). Finally, it was found that the peptides had no effect on the constitutive transcription factor Oct-1.

The effect of the PIF θ peptides on TCR-induced activation of T cells was assessed. Proliferation of primary T cells was measured after stimulation with plate-bound CD3/CD28 antibodies. As shown in Fig. 3E, a marked decrease in
30 proliferation was observed by pre-treating T cells with wild type-PIF θ peptide but not by the scrambled peptide. Reduced proliferation of T cells following treatment with wild type-PIF θ was also accompanied by a significant reduction in the level of

secreted interleukin-2 (IL-2) (Fig. 3F). The addition of IL-2 at 20 units/ml completely restored the anti-CD3/CD28 proliferative response of the wild-type PIF θ treated cells, indicating that signaling events downstream of the cytokine receptor remained intact. Proliferation of T cells after crosslinking of the TCR is a consequence of increased transcription of the IL-2 and the IL2R α chain gene, which contributes to a high-affinity receptor for IL-2. Expression of IL2R α (CD25) and CD69, a cell surface marker on activated T cells, can also be induced by treating T cells with phorbol ester. In response to anti-CD3/CD28 or treatment with PMA, the induction of both CD25 and CD69 was significantly reduced by pre-treatment with the wild type, but not the scrambled PIF θ peptide (Fig. 3G). Therefore the reduced proliferative responses of T cells after treatment with PIF θ peptide are most likely due to reduced levels of both IL-2 and CD-25-containing high-affinity IL-2 receptor.

The effects of the PIF θ peptides were also tested in vivo in a T cell-mediated cutaneous immune/inflammatory reaction to haptens, the contact hypersensitivity assay (CHS). B6 mice sensitized with dinitrofluorobenzene (DNFB) were pre-treated with PIF θ peptides for 1 hour and then challenged with hapten for 24 hours. Ear swelling induced by DNFB was significantly reduced by the wild-type peptide ($44 \pm 5\%$ inhibition), whereas the mutant had no effect (Fig. 3H). This result provides strong support for the role of PDK-1 in T-cell activation and also illustrates the potential importance of such peptides as therapies for diseases such as lupus and for immune suppression during transplantation.

Immunoprecipitation of PKC θ from Jurkat cells demonstrated that neither PI3-kinase (Ebert et al., 2000, J. Immunol. 165:543 5-42) nor LAT (Bi et al., 2001, Nat. Immunol. 2:556-63; Ward, et al., 1996, Immunol. Today 17:187-97), two upstream proteins in the TCR signaling pathway that are constitutively associated with membrane, were associated with PKC θ . Stimulation of T cells with anti-CD3/CD28 has been reported to promote PKC θ translocation to membrane rafts and establish interactions with LAT (Bi et al., 2001, Nat. Immunol. 2:556-63). Therefore, it was evaluated whether the PI3-kinase-PDK-1 pathway could lead to translocation of PKC θ to the membrane. Pretreatment of T-cells with wortmannin or LY924024 dramatically inhibited the association of PKC θ with LAT and PI3-kinase (Fig. 4A, lanes 3 and 4, respectively). Similarly, pretreatment of cells with wild type-PIF θ peptide, but not the

scrambled-peptide, inhibited association of PKC θ with LAT and PI3 -kinase (Fig. 4B, lanes 3 and 4, respectively).

Engagement of antigen-specific T cells with antigen presenting cells (APCs) has been shown to induce PKC θ translocation to the central region of the contact area between T cells and APCs, also termed central SMAC (Monks et al., 1997, Nature 385:83-6). To study the effect of PIF θ peptide on the localization of PKC θ to SMACs, in vitro differentiated Th1 cells were prepared from and TCR-transgenic mice expressing TCRs that were specific for the moth cytochrome c peptide (MCC; aa 88-103). These T cells were then labeled with PKC θ antibody and APC with 14.4.4 mouse monoclonal antibody that recognizes IE^k MHC II. Cells were pre-treated with PIF θ peptides and then incubated with non-pulsed or peptide-pulsed peptide APCs, fixed and stained for localization of endogenous PKC θ . When T cells were incubated with APCs that were not pulsed with the MCC peptide, PKC θ was localized in the cytoplasm. Stimulation with MCC peptide-pulsed APCs for 30 minutes induced PKC θ translocation to the contact area between T cells and APCs. Pretreatment with wild type-PIF θ peptides but not scrambled peptide dramatically reduced APC-induced PKC θ translocation (Fig. 4C).

Recent studies from many research groups have provided evidence that membrane rafts play an important role in T cell signaling. A variety of cytoplasmic and membrane associated proteins involved in T cell signaling have been found to be enriched in the detergent-insoluble fractions (Leitenberg et al., 2001, Semin. Immunol. 13:129-38). A recent study by Bi et al. (2001, Semin. Immunol. 13:129-38) has also demonstrated that lipid rafts are important for antigen-induced localization of PKC θ to the synapse or for conjugate formation between T cells and APCs. It was then necessary to determine whether the block of PKC θ translocation to the membrane might also affect SMAC formation and raft localization in T cells. Therefore, SMAC formation was examined in transgenic T cells incubated with the same non-pulsed or peptide-pulsed MCC peptide APCs described above. As shown in Fig. 4D, immunostaining of TCR with FITC conjugated VB3 antibody or rafts with cholera-toxin-FITC (Fig. 4E) conjugated antibody showed a clear patchy staining indicative of the formation of the SMAC and relocalization of rafts only after co-incubation of T cells with antigen-pulsed APC but not in control unpulsed APC.

Surprisingly pretreatment of T cells with wild type-PIF θ but not with scrambled peptide completely abolished SMAC formation and raft localization as determined by the sparse membrane localization of the TCR or rafts staining suggesting for the first time that the membrane translocation of PKC θ is an essential step in the formation of the SMAC.

Conflicting data in the literature have made it difficult to determine the exact mechanism by which the TCR colocalizes to the rafts during T cell activation. It has been suggested that upon engagement TCRs migrate onto the rafts (Xavier et al., 1998, *Immunity* 8:723-32), while a study by Viola et al. recently demonstrated that it is the rafts that migrate to the engaged TCR (Viola et al., 1998, *Science* 283:680-2; Viola et al., 1999, *A* 107:615-23). However, in both cases the mechanisms for raft relocation in the contact area between T cells and APCs has not been studied. However, since disruption of rafts severely affects T cell activation (Xavier et al., 1998, *Immunity* 8:723-32) and that rafts are considered important scaffold proteins for the formation of the SMAC (Xavier et al., 1998, *Immunity* 8:723-32) (Viola et al., 1998, *Science* 283:680-2; Viola et al., 1999, *Acta Path. Micro. Immuno. Scand.* 107:615-23), it is possible to hypothesize that the lack of raft migration to the membrane might inhibit the formation of SMAC. Since the data suggests that the block of PKC θ translocation to the membrane prevents SMAC formation and raft localization, the role of PKC θ may be to shuffle the rafts to the membrane and allow the recruitment of signaling components of SMAC to these domains. Consistent with this, it is interesting to note that in immature T cells, where PKC θ has been shown as not being responsible for NF- κ B activation (Sun, et al., 2000, *Nature* 404:402-7), crosslinking of CD3/CD28 fails to induce translocation of PKC θ to the rafts, possibly providing a structural basis for the different outcome of signaling in mature and immature T cells (Ebert et al., 2000, *J. Immunol.* 165:543 5-42). Taken together, the data suggest the existence of a new signaling pathway by which TCR activation through the P13-kinase-PDK-I pathway activates PKC θ -NF- κ B pathway in T cells. Pharmacological agents targeted to block of PKC θ phosphorylation by PDK-I may represent useful tools in the treatment of a wide range of immune-mediated diseases where the uncontrolled T cells activation take place. Moreover, the findings present evidence that this pathway is extremely important for the assembly of signaling components of

the SMAC shading a new light on the mechanisms by which the complex machinery of immunological synapse is regulated.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

5

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

10

CLAIMS

What is claimed is:

1. An isolated nucleic acid comprising a nucleic acid encoding a peptide fragment of PKC θ , wherein the peptide fragment selectively interacts with PDK-1.
2. An isolated nucleic acid comprising SEQ ID NO:4, wherein the nucleic acid encodes a peptide fragment of PKC θ that selectively interacts with PDK-1.
3. An isolated nucleic acid comprising SEQ ID NO:4, or a homolog, variant, mutant or fragment thereof, wherein the isolated nucleic acid encodes a peptide fragment of PKC θ that selectively interacts with PDK-1.
4. An isolated nucleic acid comprising a nucleic acid encoding a peptide fragment having at least 80% identity to SEQ ID NO:3, wherein the peptide fragment selectively interacts with PDK-1.
5. The nucleic acid of claim 1, further comprising a fusion sequence encoding a membrane-permeabilizing sequence to facilitate transport of a peptide fragment/membrane-permeabilizing fusion protein into a cell.
6. A fusion protein encoded by the nucleic acid of claim 5.
7. A method of modulating the activation of PKC θ in a cell, the method comprising administering to a cell a fusion protein encoded by an isolated nucleic acid comprising a nucleic acid of claim 5, whereby PKC θ activation is increased or decreased in the cell.

8. The method of claim 7, wherein inhibition of phosphorylation of PKC θ in the cell decreases the PKC θ activation.

9. The method of claim 7, wherein the decrease in activation of PKC θ is inhibition of activation of PKC θ in the cell.

10. A method of modulating the activation of NF- κ B, the method comprising administering to a cell a fusion protein encoded by an isolated nucleic acid comprising a nucleic acid of claim 5, whereby NF- κ B activation is increased or decreased in the cell.

11. A method of modulating the activation of a T-cell, the method comprising administering to a cell a fusion protein encoded by an isolated nucleic acid comprising a nucleic acid of claim 5, whereby T-cell activation is increased or decreased.

12. A method of treating systemic lupus erythematosus, the method comprising administering to a mammal a fusion protein encoded by an isolated nucleic acid comprising a nucleic acid of claim 5, whereby the symptoms of systemic lupus erythematosus are ameliorated.

13. A method of suppressing the immune system of an organ-transplant patient, the method comprising administering to the patient a fusion protein encoded by the isolated nucleic acid comprising a nucleic acid of claim 5, such that the patient's immune system is suppressed.

14. A method of inhibiting the T-cell receptor-induced activation of a T-cell, the method comprising administering to a mammal a fusion protein encoded by an isolated nucleic acid comprising a nucleic acid of claim 5, such that T-cell activation is inhibited in the mammal.

15. A method of inhibiting PKC θ translocation to the supramolecular activation cluster in a T-cell, the method comprising administering to a mammal a fusion protein encoded by an isolated nucleic acid comprising a nucleic acid of claim 5, such that PKC θ does not translocate to the supramolecular activation cluster.

16. A method of inhibiting formation of a supramolecular activation cluster in a T-cell, the method comprising administering to a mammal a fusion protein encoded by an isolated nucleic acid comprising a nucleic acid of claim 5, such that the components of the supramolecular activation cluster do not associate to form the supramolecular activation cluster.

17. A composition comprising a fusion protein encoded by an isolated nucleic acid comprising a nucleic acid of claim 5 and a pharmaceutically-acceptable carrier.

18. An isolated peptide comprising SEQ ID NO:3.

19. An isolated peptide that is 80% identical to SEQ ID NO:3.

20. An isolated peptide comprising SEQ ID NO:3 or any modification, variant, mutant or fragment thereof having the biological activity of interacting with PDK-1.

21. A fusion protein comprising an isolated peptide comprising SEQ ID NO:3 and a membrane-permeabilizing sequence.

22. A fusion protein comprising an isolated peptide comprising SEQ ID NO:3 or any modification, variant, mutant or fragment thereof having the biological activity of interacting with PDK-1, and a membrane-permeabilizing sequence.

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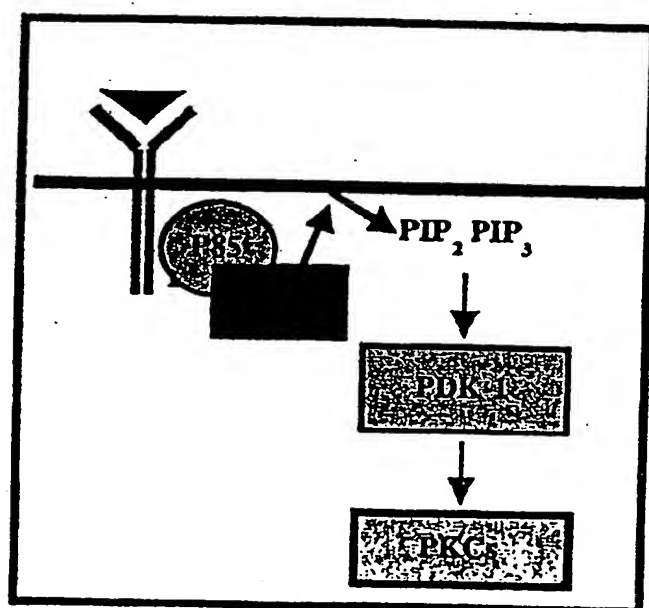


FIG.1A

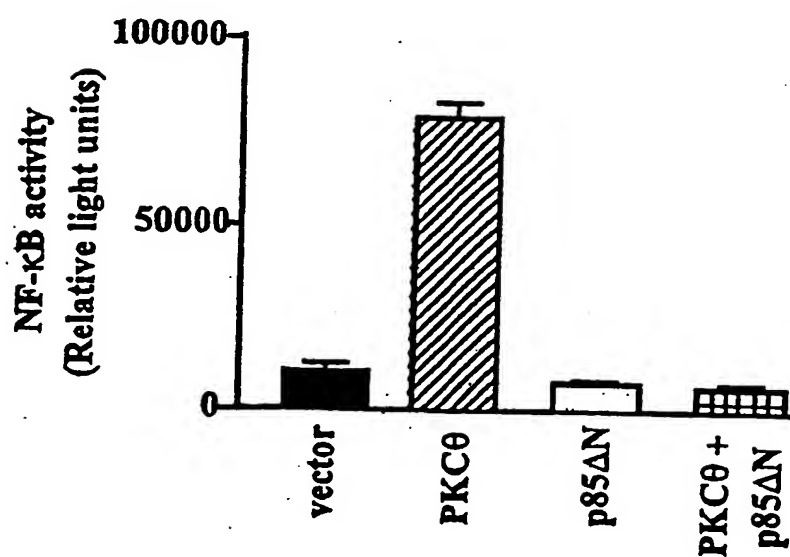
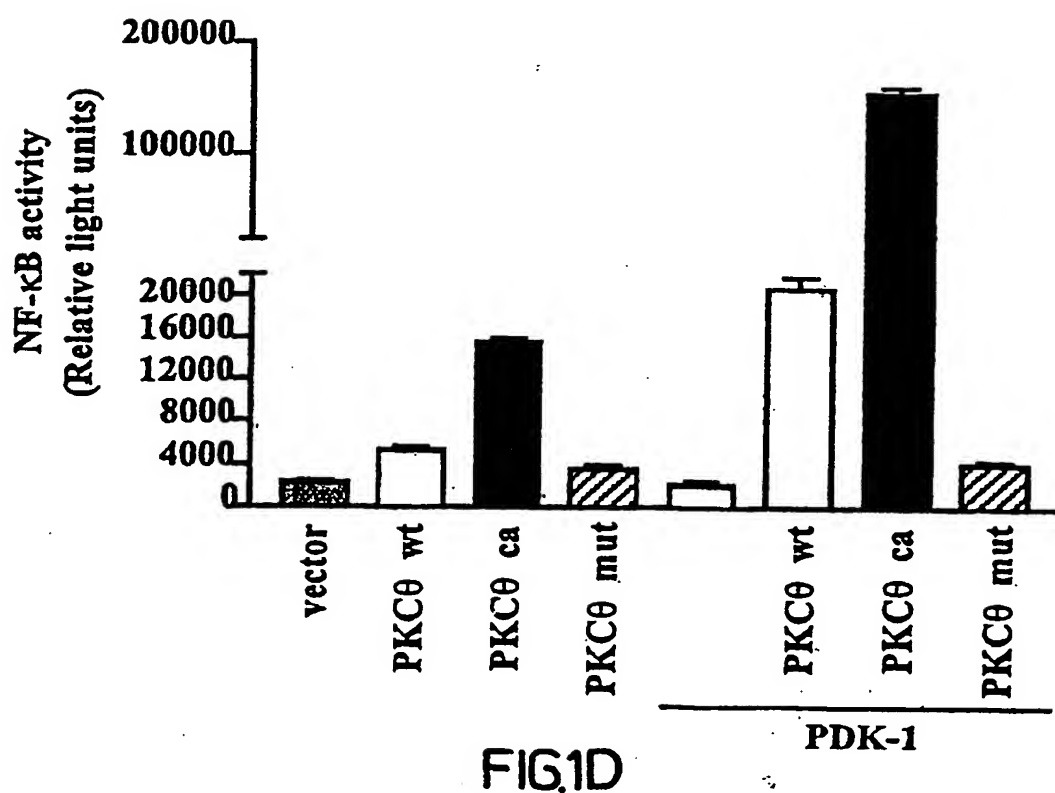
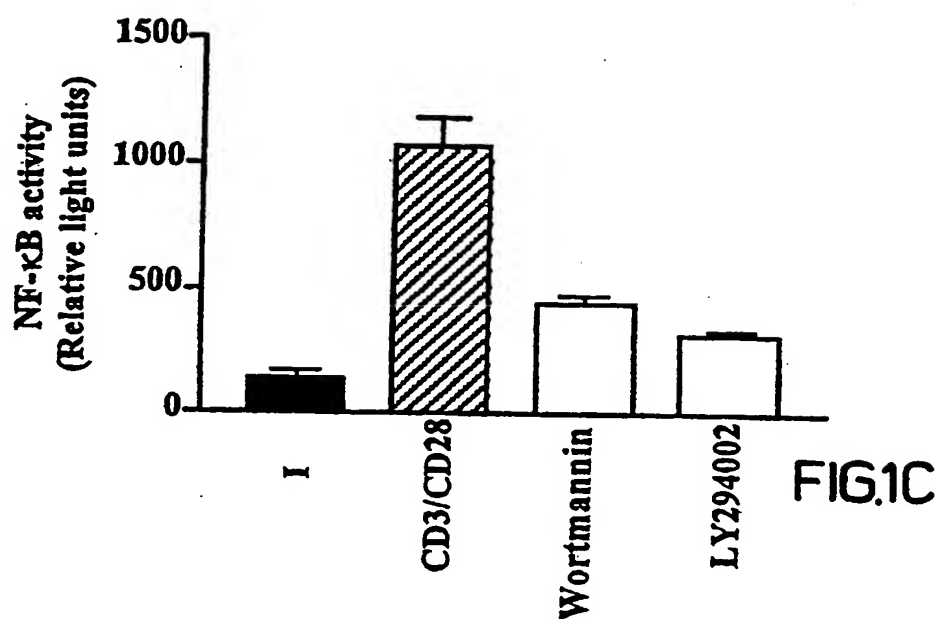


FIG.1B

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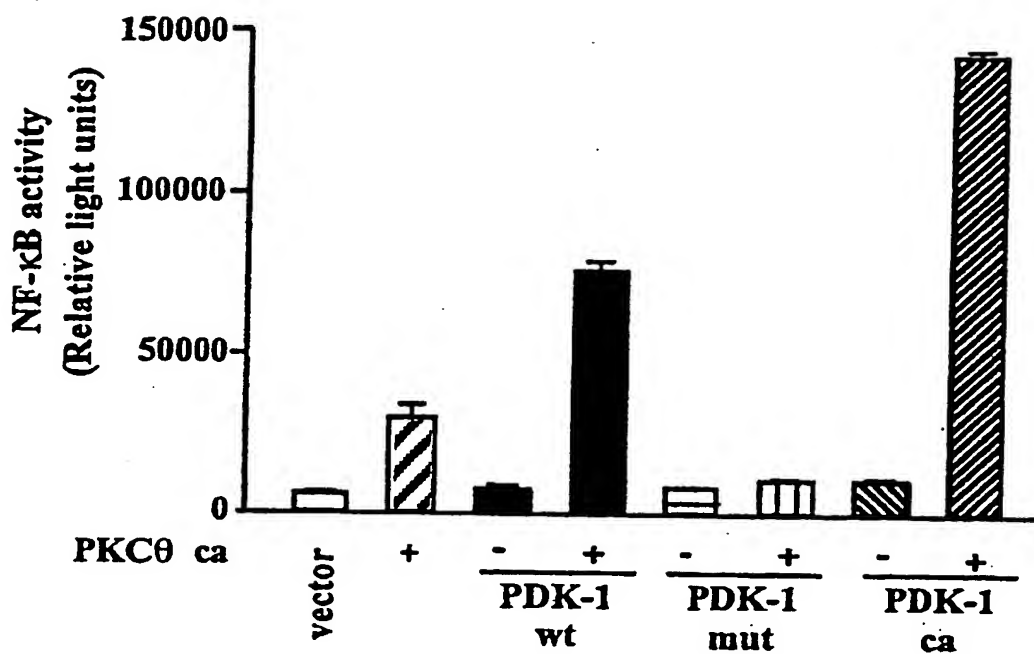
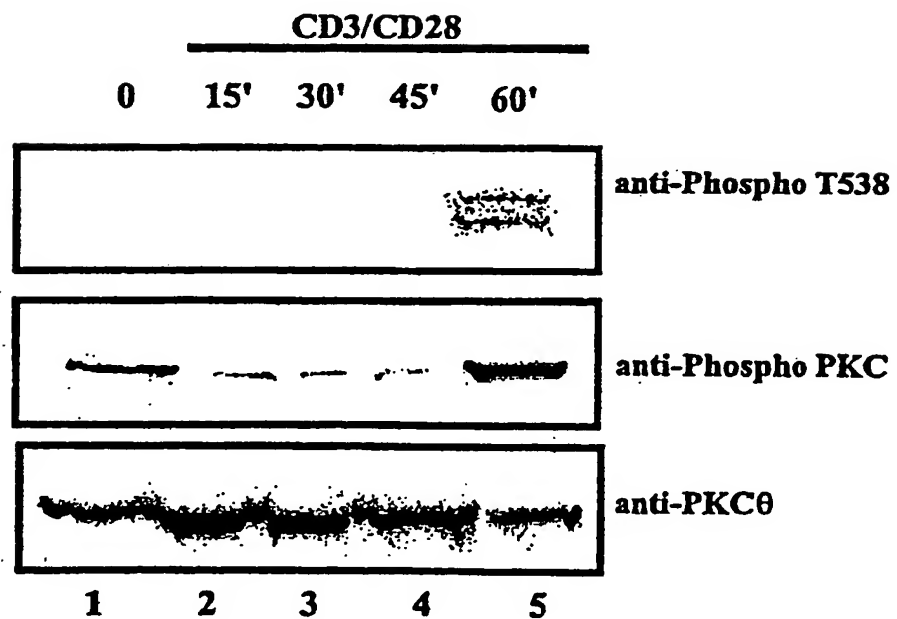
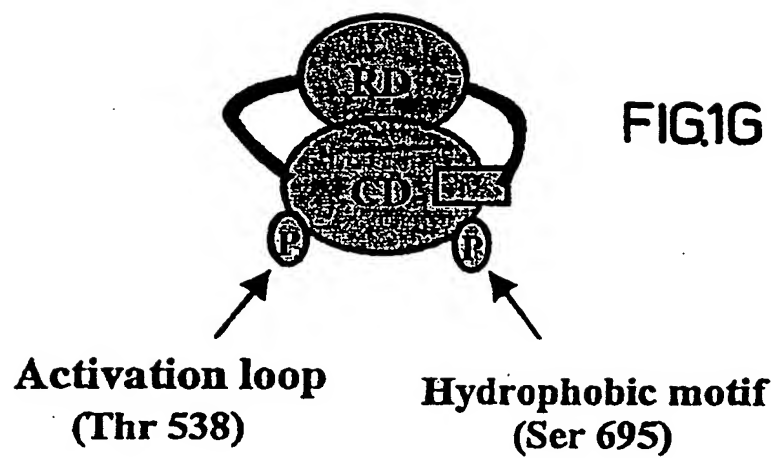


FIG.1E

PKCζ	DTTS	<u>I</u>	FCGT	FEGF	<u>E</u>	YINP
PKCδ	SRAS	<u>I</u>	FCGT	FAGF	<u>S</u>	FVNP
PKCβII	VTTK	<u>I</u>	FCGT	FRNF	<u>S</u>	FVNS
PKCθ	AKTN	<u>I</u>	FCGT	FRNF	<u>S</u>	FMNP
	ACTIVATION LOOP			HYDROPHOBIC MOTIF		

FIG.1F

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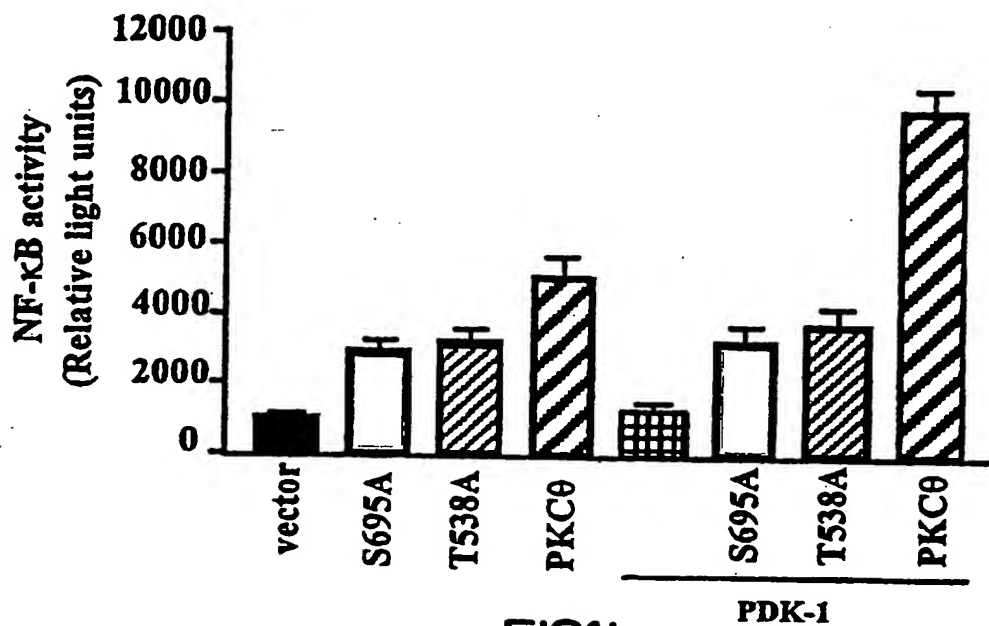


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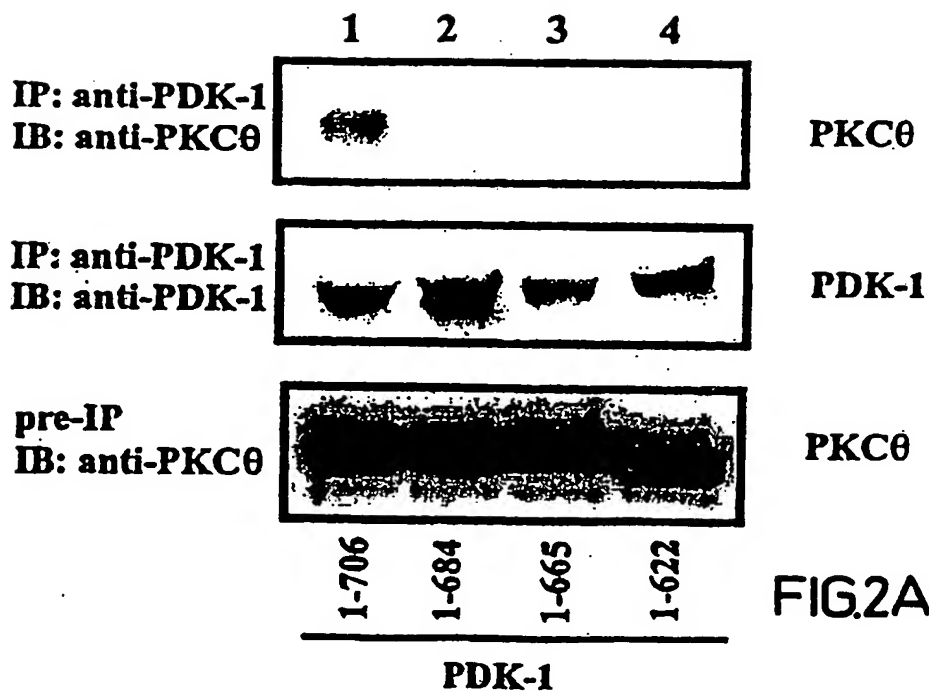


FIG.2A

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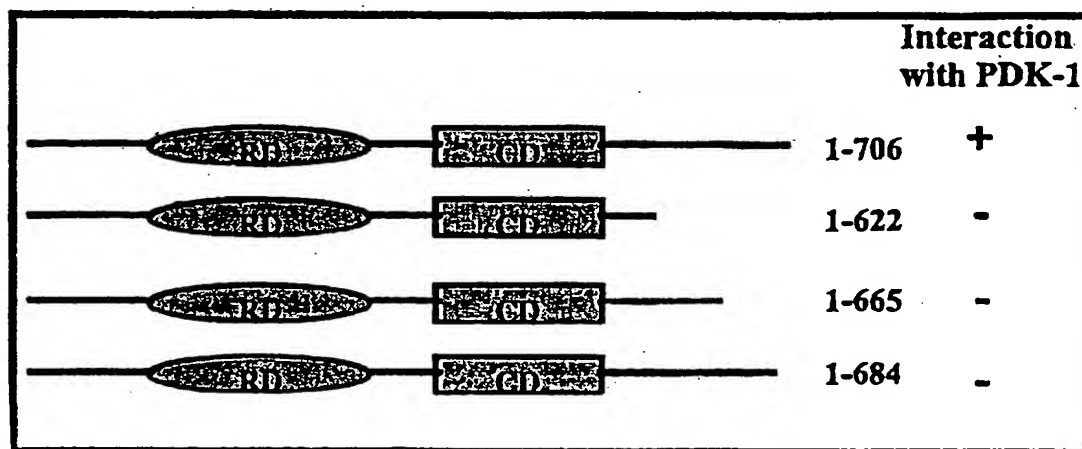


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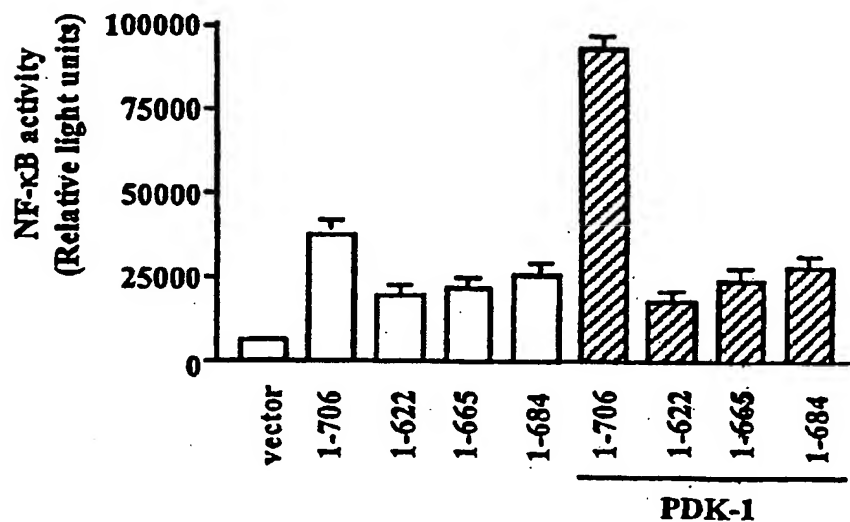
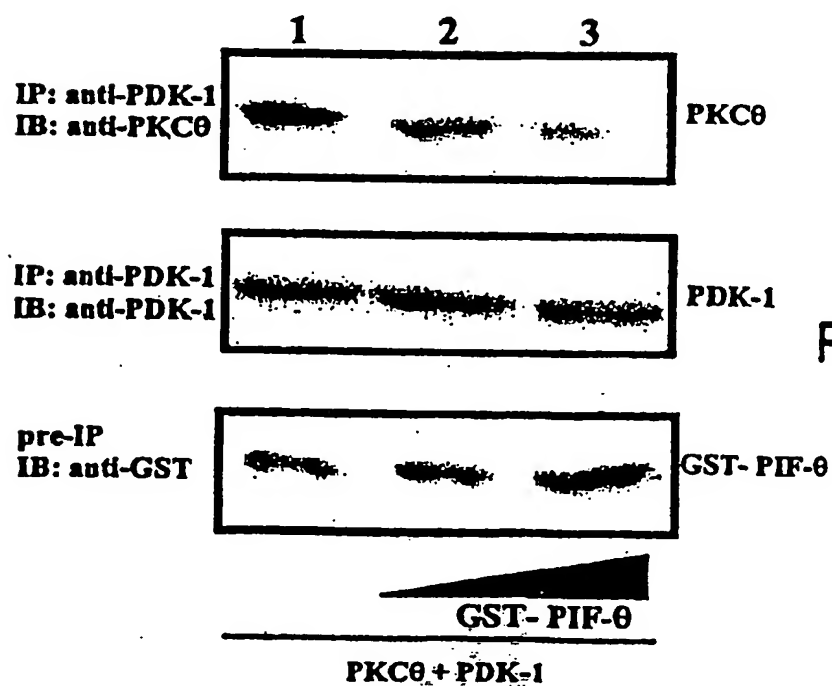
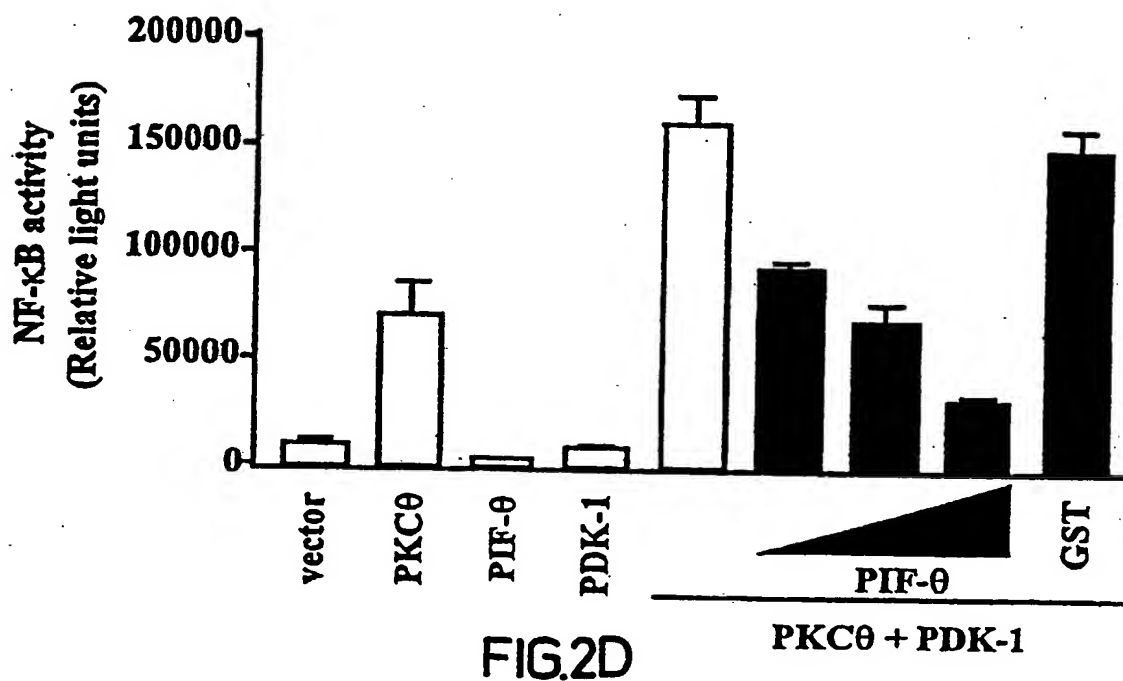


FIG.2C

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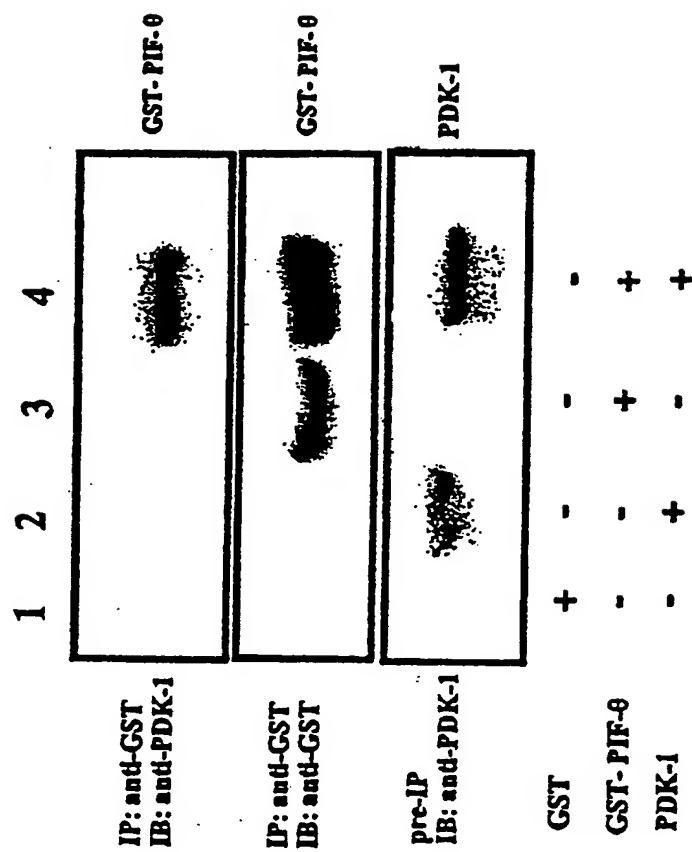


FIG.2F

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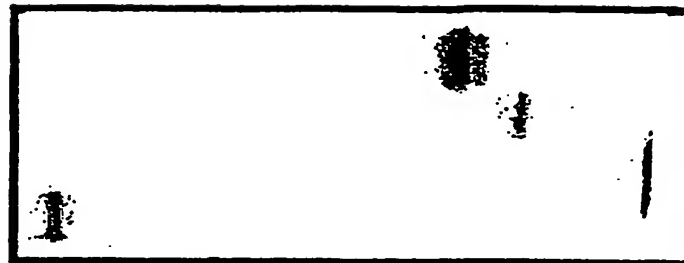


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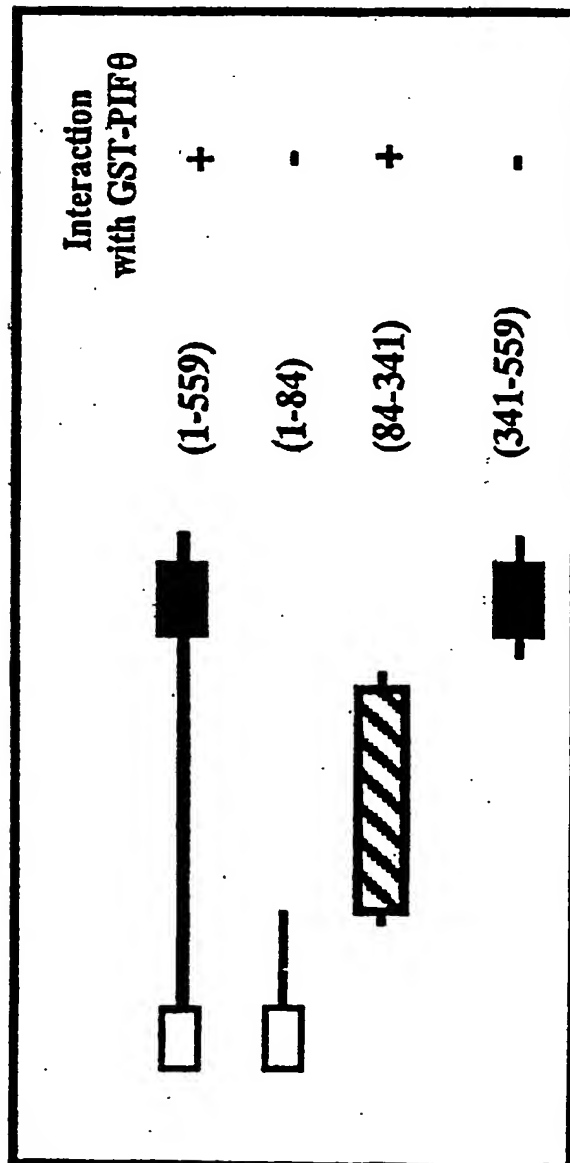


FIG.2G

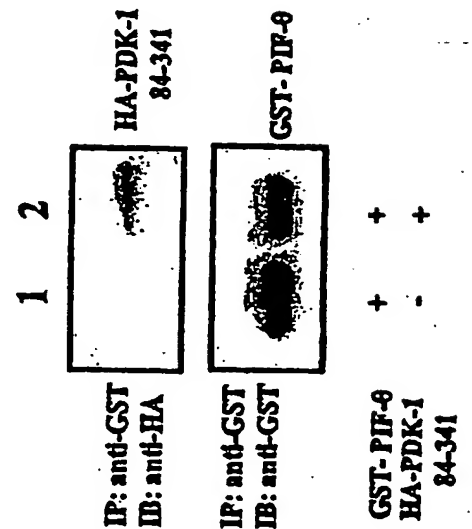


FIG.2H

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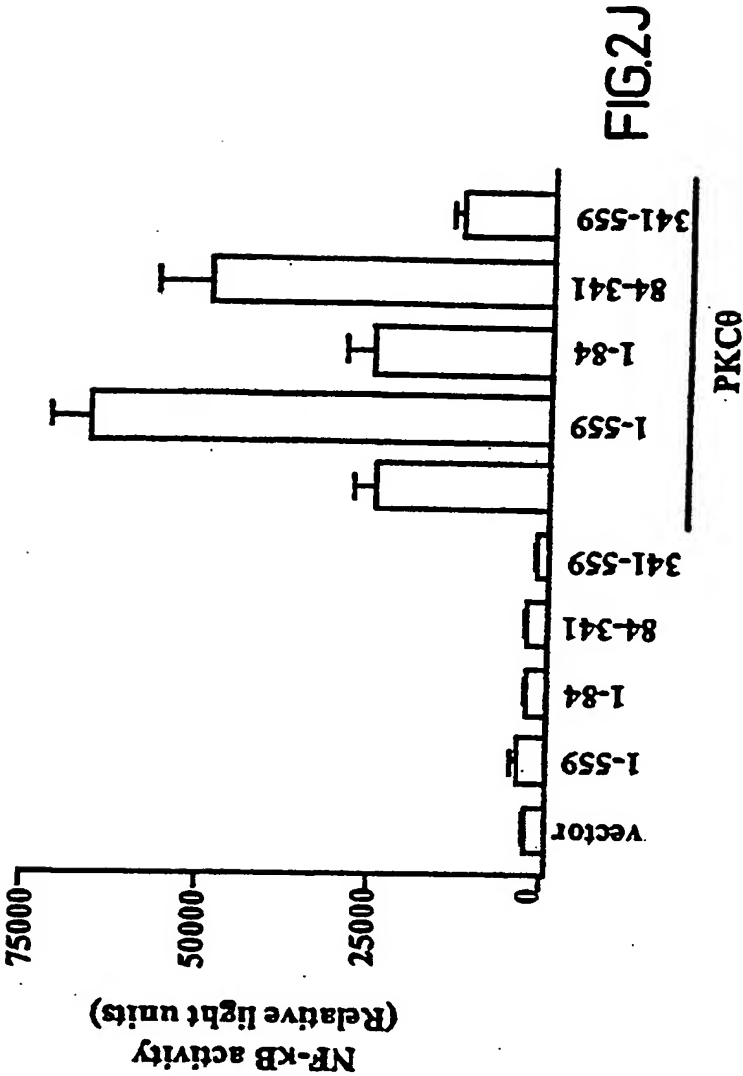


FIG.3A

wt-PIF NH₂-rqikiwfnrrrmkwkMDQNMFRNFSFMNP -COOH
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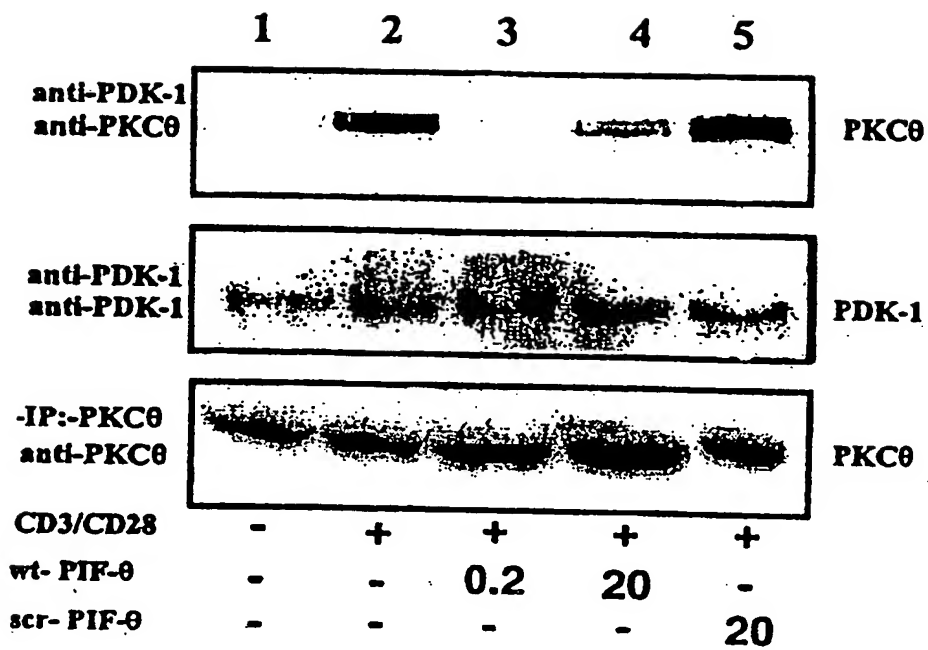


FIG.3B

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CD3/CD28	-	+	+	+	+
wt- PIF- θ	-	-	0.2	2	20



FIG.3C

CD3/CD28	-	+	+	+	+
scr- PIF- θ	-	-	0.2	2	20



FIG.3D

TNF- α	-	+	+	+
wt- PIF- θ	-	-	20	-
scr- PIF- θ	-	-	-	20



FIG.3E

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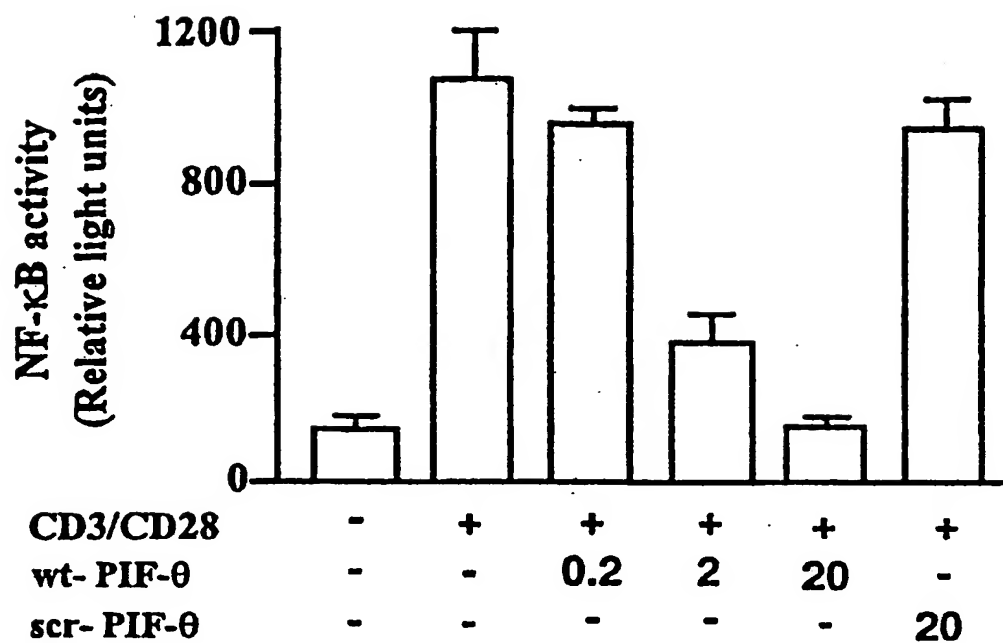


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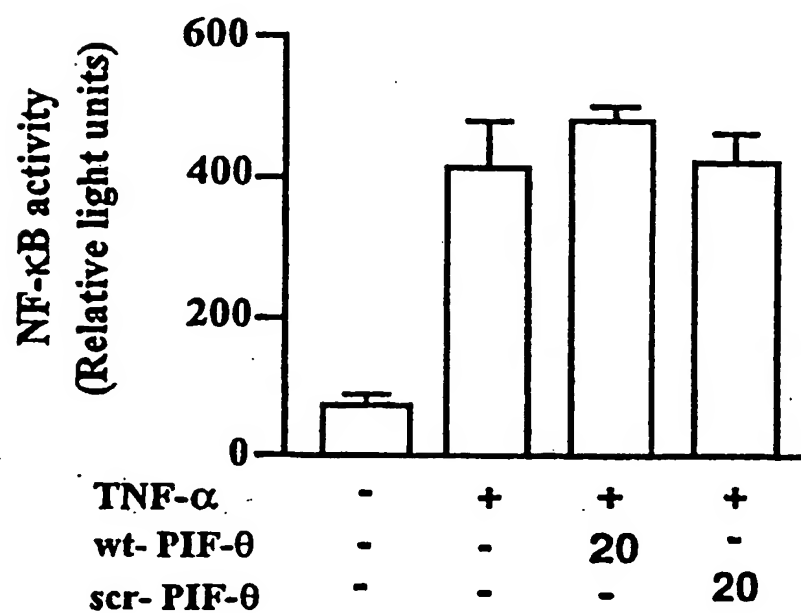
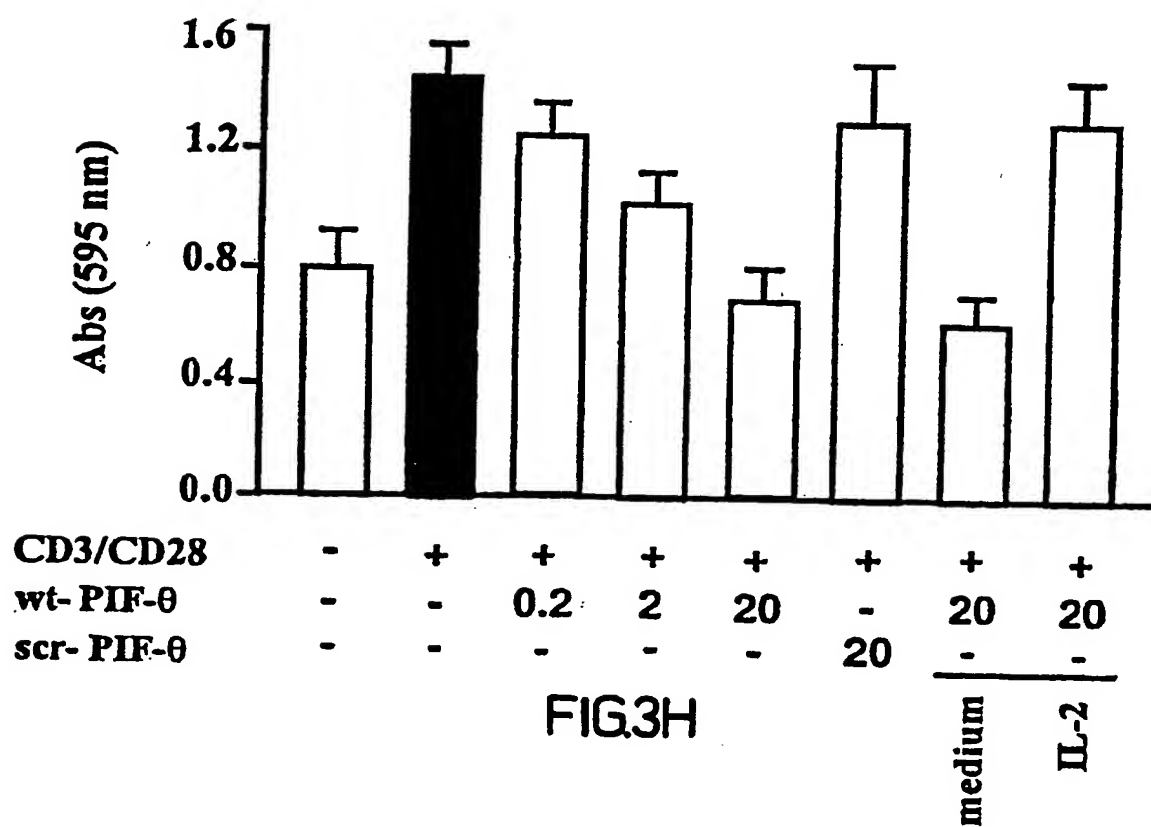


FIG.3G

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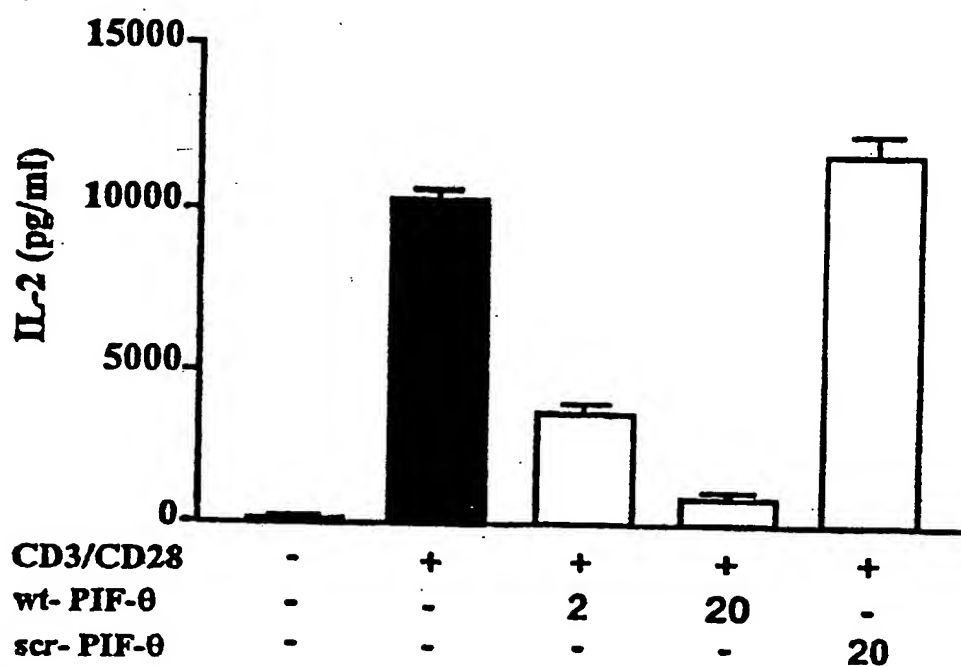


FIG.3I

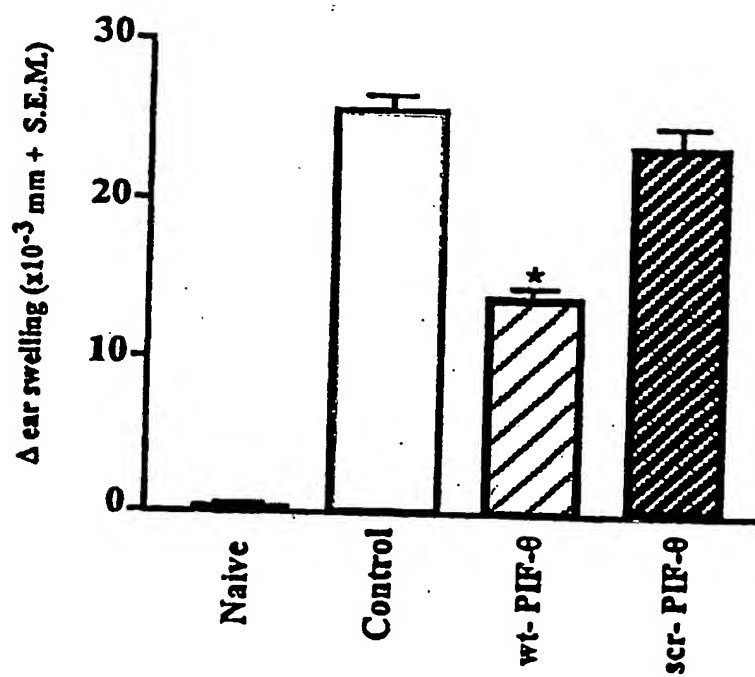


FIG.3K

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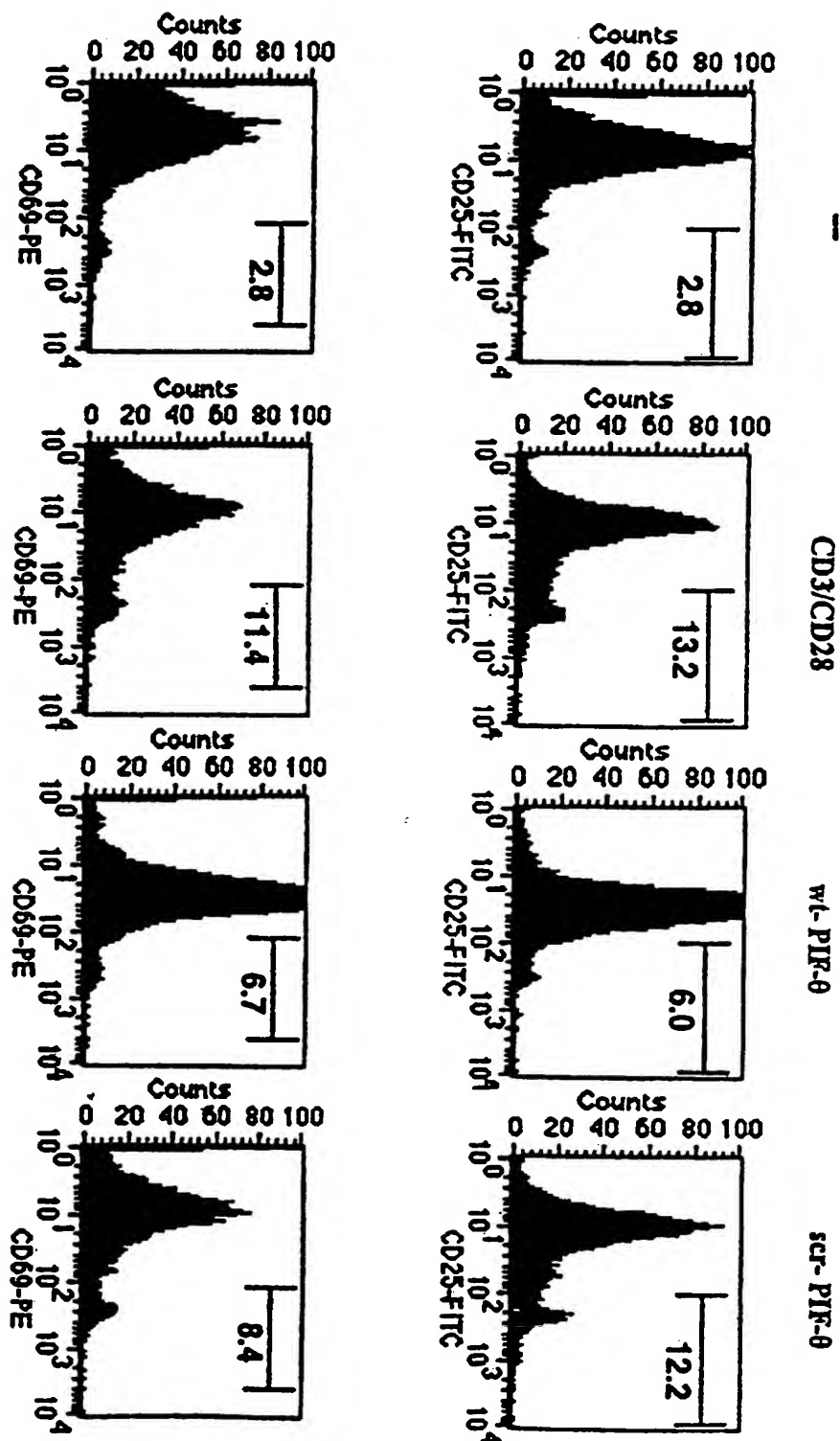


FIG.3J

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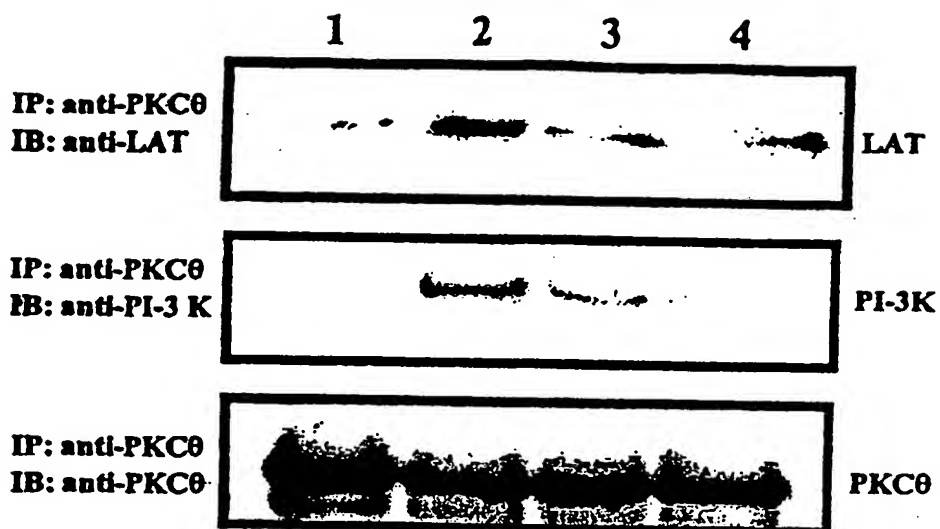


FIG.4A

I

CD3/CD28

Wortmannin

LY294002

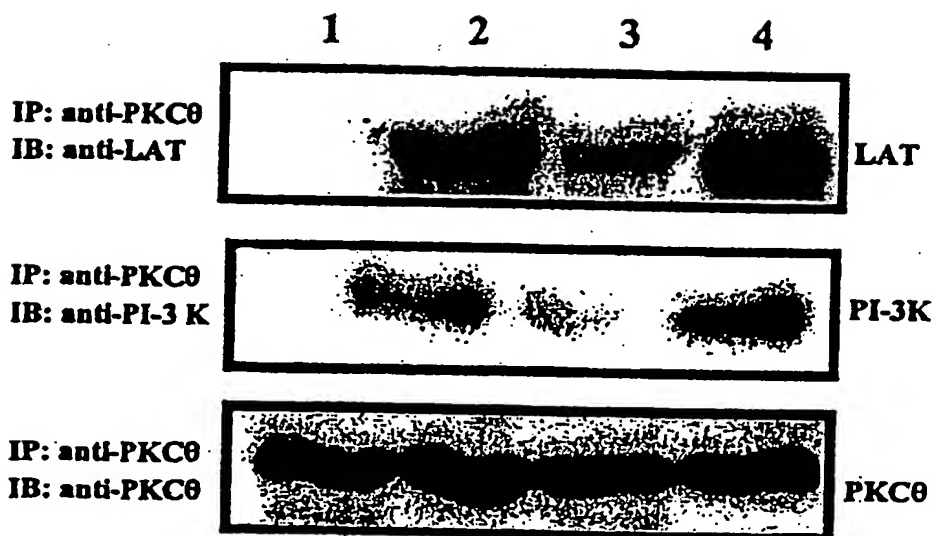


FIG.4B

I

CD3/CD28

wt-PIF-θ

scr-PIF-θ

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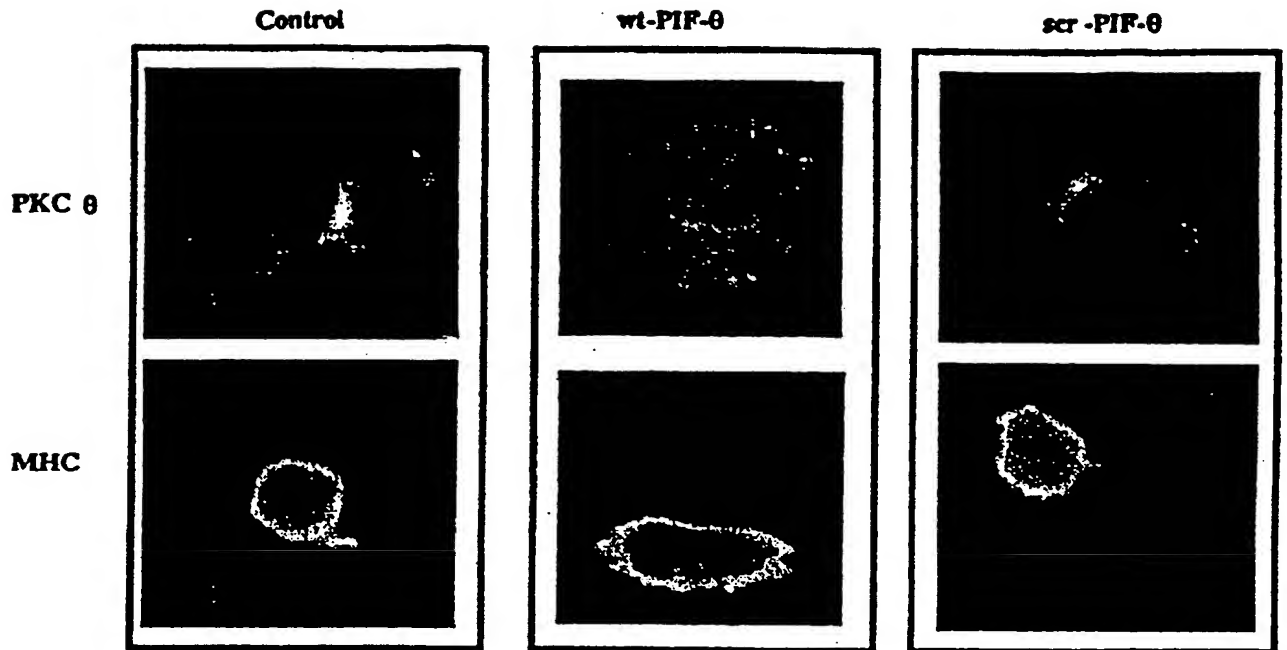


FIG.4C

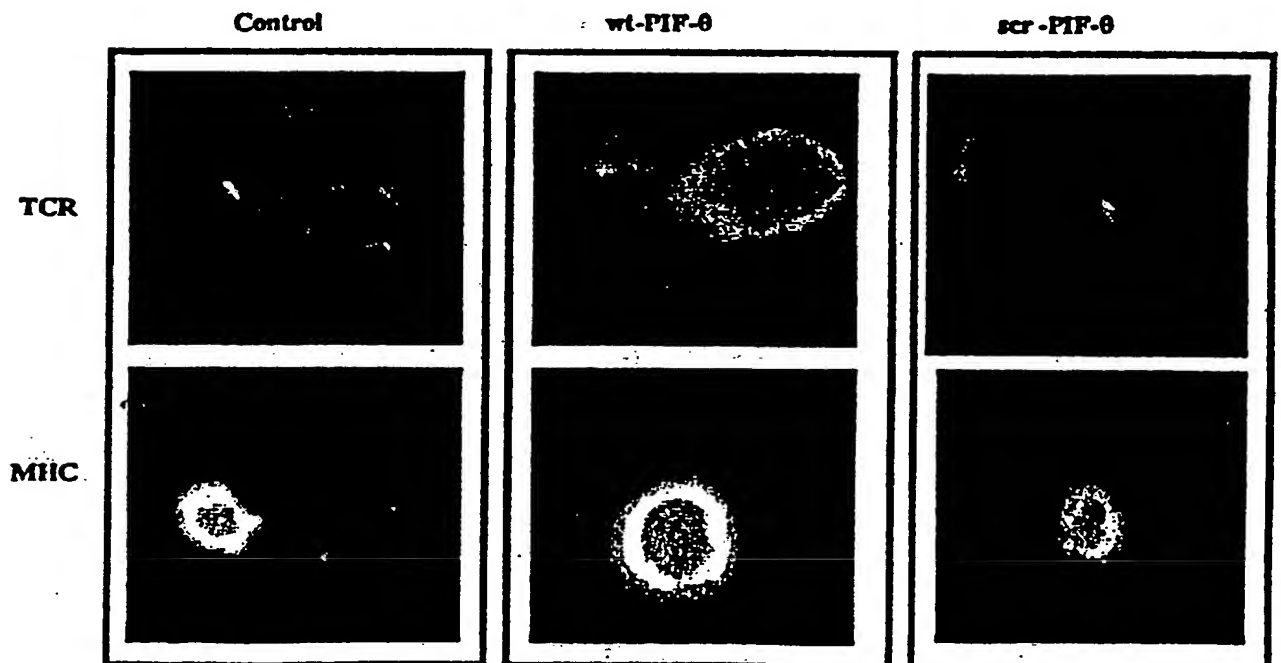


FIG.4D

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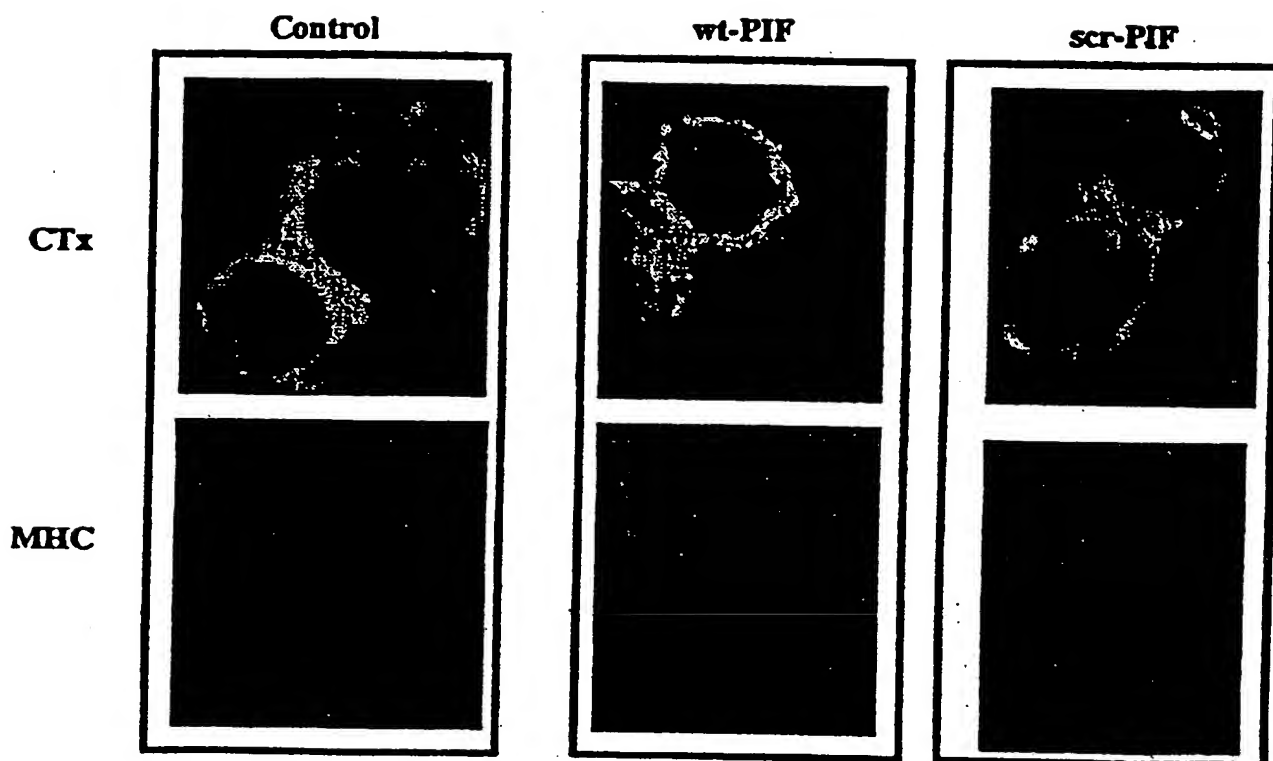


FIG4E

SEQUENCE LISTING

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GHOSH, SANKAR
FULVIO, D'ACQUISTO

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42

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PCT

(10) International Publication Number
WO 03/004612 A3

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C07K 14/00, C12N 15/63 -

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(22) International Filing Date: 2 July 2002 (02.07.2002)

(25) Filing Language: English

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60/302,474 2 July 2001 (02.07.2001) US

(71) Applicant (*for all designated States except US*): **YALE UNIVERSITY** [US/US]; Two Whitney Avenue, New Haven, CT 06511 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

(88) Date of publication of the international search report:
24 April 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **GHOSH, Sankar** [IN/US]; 9 Jonathan's Landing, Madison, CT 06443 (US). **D'ACQUISTO, Fulvio** [IT/US]; 45 Edwards Street, New Haven, CT 06511 (US).

(74) Agents: **DOYLE, Kathryn** et al.; Morgan, Lewis & Bockius, L.L.P., 1701 Market Street, Philadelphia, PA 19104 (US).

(54) Title: INHIBITOR OF T CELL ACTIVATION

(57) Abstract: The present invention provides polynucleotides, polypeptides, pharmaceutical compositions, and methods for modulation of PKC θ activation, T-cell activation, NF- κ B activation, and supramolecular activation cluster assembly and localization.

WO 03/004612 A3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/21215

A. CLASSIFICATION OF SUBJECT MATTER														
IPC(7) : CO7H 21/04; C07K14/00; C12N 15/63 US CL : 536/23.1; 530/350; 435/455														
According to International Patent Classification (IPC) or to both national classification and IPC														
B. FIELDS SEARCHED														
Minimum documentation searched (classification system followed by classification symbols) U.S. : 536/23.1; 530/350; 435/455														
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched														
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Please See Continuation Sheet														
C. DOCUMENTS CONSIDERED TO BE RELEVANT														
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.												
X	WO 01/48236 A1 (LA JOLLA INSTITUTE FOR ALLERGY) 5 July 2002 (05.07.2001). (See entire document, (SEQ ID NO:4 of the instant application is 100 % identical to SEQ ID NO:3 of cited reference).	1-22												
X	US 6,190,869 B1 (BENNETT ET AL.) 20 February 2001 (20.02.2001). (See entire document (SEQ ID NO:4 of the instant application is 100% identical to SEQ ID NO:3 of the cited reference).	1-22												
Y	SOUMITRO, P. et al. Role of protein kinase zeta in ras-mediated transcriptional activation of vascular permeability factor/vascular endothelial growth factor expression. J. of Biological Chemistry. 26 January 2001, Vol. 276, No. 4, pages 2395-2403. (See entire document, Abstract and Materials and Methods in particular).	1-22												
X	CHOU, M. et al. Regulation of protein kinase C zeta by PI 3-kinase and PDK-1. Current Biology. 1998, Vol. 8, No. 19, pages 1069-1077. (See entire document, Abstract in particular).	1												
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.														
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Date of the actual completion of the international search 12 December 2002 (12.12.2002)		Date of mailing of the international search report 31 DEC 2002												
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230		Authorized officer Michael A. Belyavskyi Telephone No. 703/308-0196												

INTERNATIONAL SEARCH REPORT

PCT/US02/21215

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BAIER, G. et al. Molecular cloning and characterization of PKC-zeta, a novel member of the protein kinase C (PKC) gene family expressed predominantly in hematopoietic cells. J.of Mol. Biol. 5 March 1993, Vol. 268, No. 7, pages 4997-5004. (See entire document, SEQ ID NO:3 of the instant application is 100% identical to sequence in the cited reference).	1-4 and 18-20

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Continuation of B. FIELDS SEARCHED Item 3:

STN, WEST, Medline, CAPlus, EMBase, Biosis, SCIssearch

Search Terms: Ghosh Sankar;D'Acquisto,F; SEQ ID NOs:3,4, PKC zeta;PKD-1; PKC

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